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EDITORIAL

Is there a place for optimizing thoracic radiotherapy in limited-stage small cell lung cancer after twenty years?

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Abstract

Thoracic radiotherapy (TRT) is one of the main treatments in limited-stage small cell lung cancer (LS-SCLC). Hyperfractionated TRT (45 Gy, 1.5 Gy twice daily) has been the standard of care (SOC) since Turrisi and colleagues published the results of their clinical trial in 1999. Two meta-analyses have demonstrated the benefits of concurrent chemotherapy and TRT in terms of intrathoracic disease control at 2 years and 3-year overall survival (OS). The phase 2 trial by Grønberg *et al* (2016) comparing once-daily hypofractionated TRT to twice-daily hyperfractionated TRT in LS-SCLC found similar outcomes in both groups in terms of response rate, progression-free survival (PFS), grade 3-4 adverse effects, and OS. The CONVERT trial, published in 2017, failed to demonstrate the superiority of the conventional scheme (once-daily TRT) *vs* twice-daily radiotherapy, despite the application of modern radiotherapy techniques and a quality assurance programme, thus confirming the twice-daily hyperfractionated regimen as the SOC. At the 2020 American Society of Clinical Oncology (ASCO) annual meeting, Grønberg *et al* reported preliminary findings from a phase 2 trial comparing two different TRT dose regimens (45 Gy *vs* 60 Gy), both administered twice daily. Those data demonstrated a marked improvement in 2-year survival rates in the high dose arm (70.2% *vs* 46.1%, $P = 0.002$), despite similar objective response rates and PFS outcomes. Those findings provide a new treatment alternative to consider: Hyperfractionated, high-dose TRT. However, the results of that trial will need to be validated in a large, randomized phase 3 study. The results of the phase 2 CALCG 30610 trial will help to clarify the optimal dose and regimen. The potential role of upfront immunotherapy, which early data suggest may improve OS, also needs to be determined.

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Core Tip: There is a lot of research regarding the role of thoracic radiotherapy in limited-stage small cell lung cancer looking for the best strategy to improve local control and overall survival. The CONVERT trial confirmed, in the contemporary era, the standard 45 Gy in 30 fractions during three weeks, concurrent with chemotherapy. High dose hyperfractionated thoracic radiotherapy in a phase II trial, presented during American Society of Clinical Oncology (ASCO) 2020, showed a better 2-year survival and a nonsignificant difference in median overall survival.

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INTRODUCTION

Small-cell lung cancer (SCLC) accounts for 13% of all lung cancers. Less than 50% of cases of SCLC present with early stage disease that include tumor node metastases (TNM) stages I through III[1,2]. Thoracic radiotherapy (TRT) combined with chemotherapy is the one of the main treatment approaches in patients with limited-stage (LS)-SCLC. This combined treatment, which has been in use since the early 1990s, has been shown to improve both local disease-free survival and overall survival (OS)[3]. Moreover, two meta-analyses comparing TRT combined with chemotherapy to chemotherapy alone, found that the combined approach improved intrathoracic disease control at 2 years (47% *vs* 24%, respectively) as well as OS at 3 years by 5.4%, thus consolidating the role of TRT in LS-SCLC[3,4].

The optimal dose of TRT has not yet been definitively established. The intergroup 0096 trial, considered a landmark study in the treatment of SCLC[5], randomized patients to receive 45 Gy in 5 wk (25 daily sessions of 1.8 Gy once-daily) or 45 Gy in 3 wk (30 daily fractions of 1.5 Gy each, twice-daily). TRT was initiated simultaneously with the first of four cycles of chemotherapy, later followed by prophylactic cranial irradiation (PCI). The median OS in the twice-daily arm was 23 mo *vs* only 18 mo in the once-daily arm. The 5-year OS in the hyperfractionated (twice-daily) group was 26% *vs* 16% in the conventional treatment group ($P = 0.04$). The local failure rate was also lower in the hyperfractionated group (36% *vs* 52%, $P = 0.06$). However, acute toxicity (grade 3 esophagitis) was higher in the hyperfractionated group (27% *vs* 11%; $P < 0.001$), perhaps due to the use of two-dimensional radiotherapy techniques with prophylactic irradiation of the mediastinum involving large volumes of normal tissue. Although twice-daily radiotherapy administered concurrently with chemotherapy was established as the standard of care, this approach was not widely used due to concerns about toxicity, logistical issues, and low doses in the control group[6].

In 2016, Grønberg *et al*[7] published the results of a randomized phase 2 trial that compared twice-daily TRT to once-daily hypofractionated TRT administered in 15 fractions. A total of 157 patients (mean age, 63 years) were evaluated. The study arms were well-balanced in terms of patient characteristics. All patients received 3D-TRT three to four weeks after the first course of chemotherapy. The therapeutic target included the pathological lesion plus elective nodal irradiation (ENI), including lymph node stations 4-7 bilaterally. The completion rates for TRT were similar, and more than 80% of patients received PCI. There were no between-group differences in response rates, or in one or two year OS rates. The median OS for the hypofractionated scheme was 18.8 mo *vs* 25.1 mo for the standard scheme [95% confidence interval (CI): 16.9-33.3; $P = 0.61$]. Grade 3-4 toxicity rates were comparable. Despite the higher complete response rate in the control arm and longer median OS, there were no between-group differences in progression-free survival (PFS), and the differences in OS were not

statistically significant. Based on these results, no firm conclusions can be drawn from this trial.

The CONVERT trial (published in 2017) was another landmark study in the treatment of LS-SCLC. This superiority trial compared concurrent once-daily to twice-daily chemoradiotherapy in patients with LS-SCLC[8]. All patients were randomly assigned to receive conformal three-dimensional-radiotherapy administered either twice daily (45 Gy in 30 fractions of 1.5 Gy) or once daily (66 Gy in 33 fractions of 2 Gy) concurrently with platinum-etoposide chemotherapy. The compliance rate was close to 90%. While most patients in both arms (approximately 80%) received an optimal number of fractions, a higher proportion of patients in the twice-daily regimen received the full radiotherapy dose. Unfortunately, the trial failed to demonstrate the superiority of the once-daily scheme; in fact, the 2-year OS was slightly higher (but not significantly) in the twice-daily arm. Acute and late radiation toxicity rates were lower than expected in both arms, perhaps due to the use of modern radiotherapy techniques and a quality assurance programme.

At the 2020 ASCO annual meeting, Grønberg *et al*[9] presented results from a phase 2 trial comparing conventional dose (45 Gy) to high dose (60 Gy) TRT administered twice daily in 30 and 40 fractions, respectively. This well-designed study was powered to show at least a 25% improvement (from 53% to 66%) in 2-year survival rates. Treatment was well-tolerated and completion rates were high (nearly 96% in both arms). There were no significant between-group differences in most grade 3 toxicities, or in treatment-related mortality. Surprisingly, the lower dose arm had a higher rate of neutropenic infections. The objective response rate was comparable in both groups, but the high dose group had a markedly better 2-year OS (70.2% *vs* 46.1%, $P = 0.002$). Preliminary findings showed a trend towards better PFS in the high-dose arm [19.9 *vs* 14.4 mo, hazard ratio (HR) 0.8, $P = 0.257$], with a significantly higher median OS in that same arm (41.6 mo *vs* 22.9 mo, HR 0.63; $P = 0.027$).

The results of that trial are highly encouraging, especially the OS outcomes, which were the best reported to date in this patient profile. However, those findings should be interpreted cautiously given that this was a phase 2 trial not stratified by prognostic variables. Moreover, the analysis was not performed in the intention-to-treat population. The two groups had a similar toxicity profile, which may be attributable to the use of modern radiotherapy techniques, treatment of involved fields, or better supportive care. However, the similar toxicity profile could also be explained by between-group differences in tumour burden, patient selection, or other unreported biases. Indeed, there are many aspects of this trial that are and will remain unknown until the full results are published, including the use of positron-emission tomography (PET) and the specific radiotherapy techniques applied. Despite the comparable response rates in the two groups, the longer survival time in the high-dose arm is noteworthy. While the reasons for this difference are not entirely clear, it is likely that other factors may be involved, such as between-group differences in patterns of relapse and/or causes of death.

Despite the growing body of evidence, the once-daily scheme (2 Gy fractions to a total of 60-66 Gy) is considered an acceptable alternative to twice-daily TRT, especially considering that some patients are unwilling or unable to undergo twice-daily treatments. Moreover, the once-daily regimen is quite common in clinics where the twice-daily schedule is not feasible; in fact, the once-daily regimen is more common in the United States[6].

In patients with LS-SCLC who are prescribed combined chemotherapy and TRT, it is generally accepted that TRT should be initiated early (together with chemotherapy or soon thereafter) and that all involved nodal stations should be irradiated. Two meta-analyses have confirmed the benefits of TRT and concurrent chemotherapy in terms of intrathoracic disease control at 2 years and 3-year OS (Table 1)[10,11].

The American Society for Radiation Oncology (ASTRO) recently published the 2020 clinical practice guidelines[12]. These guidelines, which were based on a review of the scientific evidence, recommended TRT for patients with LS-SCLC who can tolerate definitive treatment. The twice-daily scheme (≥ 45 Gy) is considered the standard of care. However, in patients who receive the once-daily regimen, the total dose should be increased to 60 Gy. The guidelines suggest that TRT should be initiated with the first or second cycle of chemotherapy; however, in certain cases, TRT can be started with the third cycle of chemotherapy. Involved field radiotherapy [fluorodeoxyglucose uptake on PET, enlarged areas on computed tomography (CT), and/or biopsy-positive areas] is recommended as the SOC. The use of highly conformal techniques is recommended to minimize radiation doses to normal tissues.

Table 1 Key studies of thoracic radiotherapy in limited-stage small cell lung cancer

Ref.	Patient (*n*)	PET	ENI	PCI	RT technique	RT timing	Dose	Chemotherapy scheme	PFS	2-yr survival	Median OS (mo)	Higher grade 3-5 adverse event
Intergroup 0096 (1999)	BID 211, OD 206	0%,0%	No	Allowed	2D (100%)	W1	45 Gy/1.5 Gy/30 F/3 wk, 45 Gy/1.8 Gy/25F/5 wk	EP: W 1, 4, 7, 10	29% 2 yr, 24% 2 yr (P = 0.10)	47%,41%	23, 19 (P = 0.04[a])	Esophagitis (55% *vs* 37%[a])
Grønberg *et al*[7] (2016)	BID 73, OD 84	0%,0%	No	84%,82%	3D (100%)	W4	45 Gy/1.5 Gy/30F/3 wk, 42 Gy/2.8 Gy/15F/3 wk	EP: W 1, 4, 7, 10	49% 1 yr, 45% 1 yr (P = 0.93)	52%,42%	25.1,18.8 (P = 0.61)	None
CONVERT trial (2017)	BID 274, OD 273	57%, 57%	No	84%,81%	IMRT (16%), IMRT (17%)	W4	45 Gy/1.5 Gy/30F/3 wk, 66 Gy/2.0 Gy/33F/7 wk	EP: W 1, 4, 7, 10, 13, 16	15.4 mo, 14.3 mo (P = 0.26)	56%,51%	30, 25 (P = 0.14)	Neutropenia (49% *vs* 38%[a])
Grønberg *et al*[9] (2020)	BID 76, BID 84	?, ?	?	82.9%, 84.5%	?, ?	W1	45Gy/1.5Gy/30F/3W, 60Gy/1.5Gy/40F/4W	EP: W 1, 4, 7, 10	14.4 mo, 19.9 mo, (P = 0.025[a])	46%,70%	22.9, 41.6 (P = 0.02[a])	Neutropenia (21% *vs* 36%[a])
CALGB 30610[1]	BID 365, OD 365		Yes	Allowed	?	W1	45 Gy/1.5 Gy/30F/3 wk, 70 Gy/2 Gy/35F/7 wk	EP o ECb: W, 4, 7, 10	Secondary endpoint		Primary endpoint	Secondary endpoint

[1]Active, not recruiting. Estimated Primary Completion Data June/2023.

[a]*P* statistically significant. BID: Twice-daily; OD: Once-daily; Gy: Grays; EP: Etoposide/cisplatin; ECb: Etoposide/carboplatin; F: Fractions; PCI: Prophylactic cranial irradiation; ENI: Elective nodal irradiation; RT: Radiotherapy; PFS: Progression-free survival; OS: Overall survival.

CONCLUSION

While the results of the large phase 2 trial by Gronberg and colleagues require validation studies, the findings suggest that it may be possible to optimize TRT through the use of high-dose hyperfractionated radiotherapy. In this regard, we are also awaiting the results of the phase 3 CALCG 30610/RTOG 0538 trial comparing chemotherapy plus standard TRT (45 Gy twice-daily) to once-daily TRT (70 Gy). That trial initially had three study arms, but one of the experimental arms was discontinued due to the findings of an interim toxicity assessment. Both TRT regimens — 70 Gy daily and 61.2 Gy concomitant boost — administered concurrently with cisplatin and etoposide chemotherapy appear to be tolerable, with no unexpected toxicities. The final results of the trial (70 Gy *vs* 45 Gy of TRT) are expected soon[13].

Several other trials are also currently underway, including a trial to assess upfront immunotherapy administered concurrently with chemotherapy plus TRT (NRG LU005 and ETOP), or immunotherapy as consolidation therapy after chemotherapy plus TRT (ADRIATIC trial). Both of these treatment approaches may be useful in this clinical setting[14], but we will have to wait for the published results to determine the true value of these treatment modalities. In the meantime, the approach used in the CONVERT trial will remain the standard of care, as this approach yields similar outcomes to those achieved in the intergroup 0096 trial by applying new imaging techniques such as PET-CT and more advanced radiotherapy techniques.

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EDITORIAL

Nonmetastatic castration-resistant prostate cancer: Novel agents to treat a lethal disease

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Abstract

Nonmetastatic castration-resistant prostate cancer (nmCRPC) - defined as prostate-specific antigen (PSA) > 2 ng/mL, testosterone castration levels < 1.7 nm/L, and the absence of metastatic lesions on conventional imaging (computed tomography or bone scan) - has been defined as a lethal disease by the Prostate Cancer Work Group. One-third of patients with prostate cancer who receive androgen deprivation therapy for biochemical recurrence after local treatment will develop CRPC, with death occurring an average of 2.5 years after diagnosis of castration resistance. Most patients diagnosed with nmCRPC are asymptomatic or minimally symptomatic at diagnosis due to local treatment. In patients with short PSA doubling times (< 10 mo) and high baseline PSA levels, there is a high risk of bone metastases followed by prostate cancer-related mortality. These patients also present significant morbidity that negatively impacts quality of life (QoL). Recently, the results of three randomized trials (PROSPER, SPARTAN, and ARAMIS) were published. Those trials evaluated the efficacy of three different androgen receptor inhibitors - enzalutamide, apalutamide, and darolutamide - in patients with nmCRPC. In all three trials, the study drugs improved both metastasis-free survival and overall survival compared to placebo, plus on-going

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androgen deprivation therapy without a negative impact on QoL. In patients with nmCRPC, the most important clinical objective is early detection and treatment to maintain a low tumor burden and to prolong the symptom-free interval. For patients with nmCRPC, these novel drugs offer new hope for better QoL and survival outcomes.

Key Words: Nonmetastatic castration-resistant prostate cancer; Prostate cancer; Apalutamide; Enzalutamide; Darolutamide; Toxicity

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Core Tip: The main focus of this editorial is to review novel treatments for nonmetastatic castration-resistant prostate cancer (nmCRPC). The three-practice changing pivotal randomized controlled trials - PROSPER, SPARTAN, and ARAMIS - are reviewed. We discuss the findings of these trials, emphasizing the strengths of the studies and critically examining controversies related to the diagnosis of nmCRPC and the role of advanced imaging techniques and molecular determinants.

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INTRODUCTION

Nonmetastatic castration-resistant prostate cancer (nmCRPC) - defined by the Prostate Cancer Working Group[1] as a prostate-specific antigen (PSA) level > 2 ng/mL and testosterone castration level < 1.7 nm/L, without evidence of metastasis on conventional radiographic imaging techniques [computed tomography (CT) or bone scans] - is considered lethal in the absence of effective treatments to extend overall survival (OS). It is estimated that 30% of patients who receive androgen deprivation therapy (ADT) for biochemical recurrence after local treatment will develop CRPC, and most of these patients will die from the disease, on average, 2.5 years from diagnosis of castration resistance.

Due to local treatment of the primary tumor, most cases of nmCRPC are asymptomatic or minimally symptomatic at diagnosis, even though most of these patients are considered "high risk" due to rapid (< 10 mo) PSA doubling time (PSADT) and high baseline PSA values. Consequently, the risk of developing bone metastases (M1) and/or prostate cancer-related mortality is high. For this reason, early identification of patients with castration resistance should be prioritized to offer effective treatments. In this regard, it is crucial to keep in mind that patients with M1 bone involvement will present substantial morbidity with a significant loss of quality of life (QoL)[2,3].

Recently, the results of three randomized trials (SPARTAN, PROSPER, and ARAMIS)[4-6] were published in high-impact scientific journals. These trials were performed to evaluate second-generation antiandrogens (enzalutamide, apalutamide, and darolutamide), known as androgen receptor inhibitors (ARIs). These agents significantly increased both metastasis-free survival (MFS) - the primary objective - and OS - the secondary objective - *vs* placebo, plus on-going ADT in patients with nmCRPC. In all three trials, adverse effects (AEs) were minimal, and QoL was excellent. These results were achieved mainly through early diagnosis and treatment, which helped to maintain a low tumor burden and to minimize symptoms in these patients for a prolonged period of time.

The emergence of these new novel agents offers new expectative to patients with nmCRPC, providing them with a remarkable opportunity for better survival outcomes (MFS and OS) while maintaining excellent QoL. In the present editorial, we review and discuss the most relevant clinical, biological, and radiographic aspects related to the diagnosis and treatment of nmCRPC.

WHY SHOULD WE TREAT NMCRPC?

Clinical evidence

The results of the aforementioned pivotal clinical trials SPARTAN, PROSPER, and ARAMIS were published between February 2018 and February 2019. The study design of these trials was quite similar, involving patients diagnosed with high-risk nmCRPC, with a rapid PSADT (≤ 10 mo) and an Eastern Cooperative Oncology Group performance status of 0-1. The patients were randomized (2:1 ratio) to receive the study drug (apalutamide, enzalutamide, or darolutamide) or placebo, plus on-going ADT. These potent ARIs prevent androgen-receptor translocation, DNA binding, and receptor-mediated transcription. Structurally, the drugs are quite similar, although with several differentiating characteristics. For example, darolutamide has less capacity to penetrate the central nervous system (blood-brain barrier) and low affinity for the γ-aminobutyric acid type A receptor, which theoretically confers a lower risk of AEs in the brain compared to enzalutamide and apalutamide[6].

The primary endpoint in all three trials was MFS. Secondary endpoints included OS, time to bone-related events, time to initiation of chemotherapy, progression-free survival (PFS), time to PSA progression, and time to pain progression, among others. The enrolment in each study ranged from 1200 to 1500 patients. Today, is it clear that MFS is an "intermediate event" (surrogate) that can predict OS. In this regard, the Food and Drug Administration has recognized that a substantial delay in the onset of metastasis is a clinically-relevant endpoint[7,8].

Although the trials are not directly comparable, they do share many characteristics: More than 70% of patients had Eastern Cooperative Oncology Group status 0, more than 70% had a PSADT < 6 mo, and only 3%-11% received bone-targeted therapies from the beginning diagnosis of nmCRPC. In addition, both the SPARTAN and ARAMIS trials - but not PROSPER - included patients with N1 disease. Only the SPARTAN study assessed PFS2 as a secondary endpoint. Treatment-related toxicity was assessed every 4 wk in the SPARTAN trial *vs* every 16 wk in the ARAMIS and PROSPER studies.

All three trials achieved the main endpoint (MFS): SPARTAN [hazard ratio (HR) 0.28; 95% confidence interval (CI): 0.23-0.35; $P < 0.0001$), PROSPER (HR 0.29; 95%CI: 0.24-0.35; $P < 0.0001$), and ARAMIS (HR 0.41; 95%CI: 0.34-0.50, $P < 0.001$). The low HR in these trials is noteworthy, as it is unusual in the field of oncology to observe such low values in three consecutive trials. These findings show that distant metastases or death decreased by 59%-72% in patients treated with the study drugs *vs* placebo. Moreover, administration of these novel agents in patients with nmCRPC extended MFS by approximately 2 years (Table 1).

The OS rates for these trials - a highly anticipated secondary objective - were recently published, showing a significant reduction in mortality risk in the treatment arms, as follows: SPARTAN, 22%; PROSPER, 27%; and ARAMIS, 31%. Median OS in patients treated with apalutamide was 73.9 mo *vs* 59.9 mo in the placebo group. The corresponding OS values for enzalutamide were 67 mo *vs* 56.3 mo. OS values have not reached for darolutamide. These findings indicate that treatment with ARIs extended OS by approximately 1 year.

Should we assess molecular determinants in nmCRPC?

In a *post hoc* analysis of the SPARTAN study, Saad *et al*[9] used the DECIPHER® platform (Decipher Biosciences Inc., San Diego, CA, United States) to evaluate the molecular subtypes associated with a decrease in PSA values and with survival outcomes. Those authors stratified patients into risk groups according to their genomic classifier (GC) scores - GC > 0.6 (high risk) *vs* GC ≤ 0.6 (low-to-average risk) and also stratified patients according to the molecular subtype (basal or luminal A/B). The biomarker characteristics of these patients revealed the presence of aggressive disease. A total of 116 patients (50%) had a GC > 0.6 (high risk), 151 patients (65%) had a basal subtype *vs* 8 patients with the luminal A subtype. Importantly, apalutamide improved survival outcomes in all patients, regardless of the specific molecular subtype. Patients treated with apalutamide plus ADT presented a rapid and constant decrease in PSA levels, regardless of the specific molecular characteristics (high or low-average GC, basal/luminal subtype). The patients who presented the largest decrease in PSA levels were those with a low-risk GC profile and those with the luminal subtype. Treatment with apalutamide plus ADT improved both MFS and OS compared to placebo in all molecular subtypes, although the greatest benefits were observed in those with high-risk GC and the luminal subtype.

Although there is not strong evidence to support that earlier initiation of ARIs in

Table 1 Metastasis-free survival in patients with nonmetastatic castration-resistant prostate cancer in the SPARTAN, PROSPER, and ARAMIS randomized controlled trials

Objective: MFS	*n*	HR for MFS	95%CI; *P* value
Apalutamide *vs* placebo (SPARTAN)	1207	0.28	0.23-0.35; < 0.0001
Enzalutamide *vs* placebo (PROSPER)	1401	0.29	0.24-0.35; < 0.0001
Darolutamide *vs* placebo (ARAMIS)	1509	0.41	0.34-0.50; < 0.001

CI: Confidence interval; HR: Hazard ratio; MFS: Metastasis-free survival.

patients with nmCRPC may result in a more aggressive molecular subtype or clinical stage, it is a controversial issue.

Smith *et al*[10] evaluated the frequency of AR anomalies, including AR-V7 expression, AR mutations, and AR amplification, before and after treatment with apalutamide plus ADT. In addition, they examined the impact on the exploratory endpoint PFS2 and time on subsequent therapy in patients with nmCRPC who were enrolled in the phase 3 SPARTAN study. Frequency of total AR anomalies was higher in patients at end of treatment than at baseline for both the apalutamide and placebo groups (apalutamide *vs* placebo at baseline: 16% *vs* 13% and at end of treatment: 20% *vs* 30%). Most patients with AR anomalies in the apalutamide group achieved a PSA response at 12 wk. Following the end of apalutamide or placebo treatment, individual AR anomalies did not have a substantial effect on PFS2.

There is a clear need to determine molecular subtypes in prostate cancer to identify better the clinical subgroups likely to benefit from specific therapies[11].

Do apalutamide, enzalutamide, and darolutamide have different toxicity profiles?

To date, no head-to-head randomized studies have been performed to compare these three agents, although the available evidence clearly shows that all three have a relatively safe toxicity profile. The overall rate of grade 3/4 AEs in the three trials was approximately 24%, with the most common AEs being hypertension, fatigue, erythema, falls, fractures, and cognitive alterations. The rates of AEs reported seem to be lower in the darolutamide study. Apalutamide has a unique rash reported in 23.8% of patients (grade 3-4 4.2%). Most of them were solved, allowing to continue treatment. The discontinuation rate due to treatment-emergent AEs was quite low, ranging from 8.9% to 15% (apalutamide 15%, enzalutamide 9.8%, darolutamide 8.9%), and few treatment-related deaths have been reported.

Selection of the most appropriate first-line drug will depend on the clinical, biological, and logistical factors at the individual treatment center as well as on availability and cost of the agent, among other factors. In other words, selection will be personalized according to the specific needs of each patient.

Have any meta-analyses been performed for nmCRPC?

Two study level meta-analyses of the three randomized trials have been performed to date[12,13], the most recent being the meta-analysis conducted by the Toronto group, presented at the American Society of Clinical Oncology 2020 meeting[12]. In that meta-analysis, the authors evaluated survival outcomes and treatment-related toxicity in the three trials, finding a clear benefit for all three agents *vs* placebo for MFS (HR 0.32; 95%CI: 0.25-0.41), PFS (HR 0.08; 95%CI: 0.05-0.13), and OS (HR 0.74; 95%CI: 0.61-0.90). In terms of grade 3-4 toxicity, treatment with these agents was associated with significantly more AEs than placebo (HR 1.47; 95%CI: 1.27-1.71).

Does local treatment provide any benefit in nmCRPC?

There is no solid evidence that treatment (radiotherapy or surgery) of the primary tumor has any benefit in patients with PCa who subsequently develop nonmetastatic castration-resistance, although the findings of the STAMPEDE trial[14] demonstrated that hormone-sensitive metastatic patients with a low tumor burden who received radiotherapy presented better OS outcomes than untreated patients.

A *post hoc* analysis of the SPARTAN trial[15] assessed the impact of initial radical local treatment on OS. In patients who received local treatment, the hazard ratio for OS was better in the apalutamide group (HR 0.67; 95%CI: 0.45-0.98), with a clear and consistent separation between the apalutamide and placebo groups on the Kaplan-Meier OS curves after 15 mo. In the subset of patients who did not receive radical local

treatment, although the HR was still favorable for the apalutamide group (HR, 0.82), it was not statistically significant.

The mechanism by which local treatment impacts survival in patients with metastatic PCa is unknown. A plausible mechanism is that eradication (or debulking) of the local tumor may prevent future metastases from the primary tumor. Although this hypothesis should be interpreted cautiously, there is enormous interest in determining the value of definitive local treatment in patients with nmCRPC, an approach that warrants investigation in prospective studies.

What role do new imaging techniques play in patients with nmCRPC?

Conventional imaging tests - CT, bone scans, and pelvic magnetic resonance imaging - were performed in all three trials, and the overall evidence suggests that conventional imaging techniques are adequate. However, newer imaging modalities such as choline ^{68}Ga-prostate-specific membrane antigen positron emission tomography (PSMA-PET)/CT have greater sensitivity and specificity than conventional imaging tests[16,17] and are capable of detecting metastatic lesions that are not visible on conventional imaging. These more advanced imaging techniques could potentially provide important data to improve decision-making. In fact, the RADAR III group[18] recently concluded that next-generation imaging techniques should be used in patients with nmCRPC who present a rapid (< 6 mo) PSADT, provided that there is an appropriate treatment available to treat the metastases and alter the course of disease.

The most relevant data published to date were reported by Fendler *et al*[19], who retrospectively evaluated 200 patients with high-risk nmCRPC. In that study, the authors centrally reviewed data obtained by PSMA-PET imaging to determine the detection rate for pelvic disease and distant metastases, which was positive in 196 of the 200 patients evaluated. Overall, despite negative conventional imaging studies, PSMA-PET imaging revealed pelvic involvement in 44% of the sample (24% with local prostate bed recurrence) and M1 disease in 55%.

QoL in nmCRPC

All three trials evaluated treatment-related QoL. Overall, the findings from these three trials indicate that, in asymptomatic patients with high-risk nmCRPC, treatment with ARIs does not appear to impact negatively health-related QoL[20]. In recent years, the association between second-generation antiandrogens and health-related QoL has been assessed not only in nmCRPC but also in hormone-sensitive metastatic prostate cancer. The available data strongly support the use of these drugs, which have proven efficacious with acceptable treatment-related toxicity that does not negatively impact QoL. In short, the introduction of these new agents has generated a revolutionary impact in the treatment of patients with nmCRPC.

CONCLUSION

To conclude, nonmetastatic CRPC is a heterogeneous, aggressive, and lethal clinical disease. If left untreated, most high-risk nmCRPC patients develop significant symptoms with a substantial deterioration in QoL, leading to a rapid death. However, the published data from clinical trials have shown that treatment with these novel ARIs (enzalutamide, apalutamide, and darolutamide) significantly improves MFS, OS, and QoL. Moreover, all of these agents have a low toxicity profile and can therefore be used safely.

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ORIGINAL ARTICLE

Observational Study

Evolutionary model of brain tumor circulating cells: Cellular galaxy

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Abstract

BACKGROUND

Although circulating tumor cells (CTCs) have been the focus of consideration for a decade, a categorized cell-based diagnostic strategy is unavailable. The personalized management and complementary/analytical-strategy of data require an alphabetic guide. Therefore, we aimed to determine the behavior of CTCs in tumor and blood in order to provide the hypothetical-based agenda in the brain neoplasms. Exploring the protein expression (PE) using a single cell-based method would clarify the heterogeneity and diversity in tumor and blood, which are key events in the evolution in brain tumors. In fact, heterogeneity, diversity, and evolution are required for cancer initiation and progression.

AIM

To explore CTCs in brain tumors and blood cells and to assay intensity of PE through personalized insight.

METHODS

The focal population included 14 patients with meningioma, and four patients with metastatic brain tumors (T). PE was assayed by immunofluorescence in tumors cells and CTCs in 18 patients with brain tumors. Ratio test was applied between the T cells and CTCs in tumor tissue and in vascular system. T/CTC ratio-based classification of PE in macrophage chemoattractant chemokine ligand 2 (CCL2), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), CD133, cyclin E, neurofilament marker, cytokeratin 19, and leukocyte common antigen (CD45) were investigated.

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RESULTS
Total analyzed cells ranged between 10794-92283 for tumor cells and between 117-2870 for CTCs. Characteristics of histopathologic and status of an ataxia-telangiectasia mutated polymorphism (D1853N) in 18 patients affected with brain tumors were also provided. The course of evolution and metastatic event relied on the elevated protein expression in CTCs, which could be considered as a prognostic value. Diverse protein expression of the migrated cells into the blood stream and the tumor was indicative of the occurrence of evolution. Besides, the harmonic co-expression between CCL2/EGF and CCL2/VEGF could facilitate the tumor progression including the metastatic event. Expression of these proteins in the migrated vasculature and into the buccal tissue offered a non-invasive follow-up detection in neoplastic disorders. PE-exploration of neurofilament marker/CD133/VEGF of the CTCs in meningioma and cytokeratin 19/CD45/cyclin E in the patients with metastatic brain tumor would clarify the tumor biology of the brain neoplastic disorders.

CONCLUSION
The alphabetical base of the evolutionary mechanisms relies on dual-, triple-, and multi-models with diverse intensity of expression. In fact, cross-talk between initiative and the complementary channels defines the evolutionary insight in cancer. A diverse-model of protein expression, including low, medium, and high intensity, is the key requirement for the completed model. The cluster of cells with diverse expression and remarkable co-expression between CCL2/EGF/VEGF and NM/CD133/VEGF in CTCs may be indicative of probable invasiveness of the tumor. Furthermore, the mode of cytokeratin-19^{+}/CD45^{-} can be traced in the metastatic patients.

Key Words: Circulating tumor cells; Brain tumor; Protein expression; Personalized; Somatic/circulating tumor cell evolution; Metastasis

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Core Tip: The present data revealed that a classified/functional/individualized cell-based preventive and therapeutic strategy is required for the cancer management. An interaction between chemoattractant chemokine ligand 2/epidermal growth factor/vascular endothelial growth factor is an influential and key utility in the approach of cancer cells. Tracing the tumor/circulating tumor cells evolution will facilitate the estimation of functional alterations in blood stream as a model to predict the probable initiative step for metastasis, formulating the progressive status of disease and by planning for a translational/personalized management. Finally, heterogeneity and diversity may minimize the severity of disease.

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INTRODUCTION

Based on the World Health Organization system, meningiomas are classified as the second most common-primary brain tumor (T) of the central nervous system in adults. Brain invasion has been included in the diagnostic criteria as atypical meningioma World Health Organization grade II[1]. The pitfalls in neoplastic diagnosis include late detection and unavailability of the precise formulated, classified data, the somatic/circulating tumor cells (CTCs)-based heterogeneity and evolutionary process. Three elements, including angiogenesis, edema, and growth, play a key role in the progression of meningioma. The vascular system of meningioma is rather diverse, extending from sporadic vascularization to the vast mode, like angiomatous meningi-

oma[2].

Chemoattractant chemokine ligand 2 (CCL2/C) is involved in the process of cell migration and plays a key role in regulation of the transferring process through blood brain barrier (BBB)[3]. Macrophages are an influential factor in metastatic programming[4]. Besides, monocytes play a vital role in the seeding process through angiogenesis, so there is a cross-road between CCL2, macrophage, monocytes, and vascular endothelial growth factor (VEGF/V)[5]. However, such configuration is required for a successful event in the developmental seeding process of metastasis. Furthermore, no characterization of the expression on the "human cerebrovascular chemokine" has been provided[6,7].

VEGF is a key factor for formation, growth, development, and survival of tumor through angiogenesis. VEGF is a remarkable factor associated with the diverse potential of angiogenesis and metastasis in human neoplasms, but the precise functional mode of VEGF is not well known in meningioma. However, a remarkable vascularization related to the vascular permeability factor in some patients with meningioma has been reported[2]. Angiogenesis is essential for progression and the metastatic process[8]. However, it is required to unmask the diverse spectrum of protein expression (PE) in meningioma. This is a hypothesis on the puzzling quality and quantity of VEGF expression.

Epidermal growth factors (EGFs/E**)** are mainly involved in differentiation, cell proliferation, tumorigenesis, and apoptosis. An association has been found between high expression of EGF and progression, development, invasiveness, metastasis, and poor prognosis in cancer[9-12]. Furthermore, the intravasation/migration to the blood stream (BS) is required for "seed and soil" theory[13].

Since CTCs were first observed in a patient with metastasis in 1869, this strategy has not been considered a routine diagnostic tool[14]. The biological aspects of CTCs in metastasis have been highlighted previously[15]. It was stated that the invasiveness of CTCs could be related to their characteristics[16]. The CTCs from glioma had no metastatic behavior in the peripheral territory after migration to BS at the microRNA level[17]. Besides, the liquid biopsy for the deoxyribonucleic acid-sequencing-based test was published[18,19]. The CTCs have also been evaluated as a predictor of survival in patients with melanoma[20]. However, metastasis is characterized by the sequential puzzling events with diverse behaviors in different cancers.

The aims of this study were to: (1) Trace the PE diversity in CCL2/VEGF/EGF; and (2) Classify the PE behavior of these proteins at the somatic/CTC level to provide the personalized strategy for the early detection of CTCs. Upon these aims, the preventive/ predictive tasks are provided as a personalized model by exploring eight markers in patients with brain tumors. This strategy offers the initial step of the cell-based/formulated/categorized model for an early diagnosis of probable metastatic process and the follow-up plan through the patients' life.

MATERIALS AND METHODS

Study population and data collection

Patients and clinical information: The focal population included 10794-92283 cells for tumor cells and between 117-2870 for circulated T cells, which were analyzed in 18 patients, including 14 with meningioma and four patients with metastatic brain tumors [one with lung-, two with breast-origin, and one with cerebellar medulloblastoma (CM)] (Table 1). The samples were referred from the Department of Neurosurgery, Shariati Hospital, Tehran University of Medical Sciences (TUMS), and the consent letter was signed before surgery. Peripheral blood (PB) and tumor samples of patient were investigated at the Department of Medical Genetics, TUMS.

Study design

Design of this investigation was based on evolutionary and personalized insights.

Analytical strategy

The analytical strategy (AS) was designed as a complementary tool for more precise/specific analysis and categorizing the achieved data. Diverse intensity of PE was categorized as C1, C2, and C3 for CCL2; V1, V2, and V3 for VEGF; and E1, E2, and E3 for EGF.

AS is based on the intensity of expression, which varies in different proteins and is provided as C1 (patient 5), V1 (patients 1 and 5), E1 (patient 5); C2 (in patients 5 and 8), V2 (patient 5), E2 (patient 5); and V3 (patient 4). An equal ratio is, exceptionally,

Table 1 Characteristics of histhopathologic and status of ataxia-telangiectasia mutated polymorphism (D1853N) in patients with brain tumors

Patient ID	Age in yr	Histopath/grade	Other complications	D1853N polymorphism status
1	46	Meningiotheliomatous type	-	+
2	47	Meningioma/I	-	-
3	34	Meningothelial type	-	+
4	40	Meningiotheliomatous type	Focal micro-invasion to the underlying brain tissue	+
5	37	Meningioma/angiomatous	-	-
6	66	Meningothelial type	-	-
7	56	Meningothelial type	-	
8	50	Meningiotheliomatous type	-	-
9	21	Meningiotheliomatous type/I	-	-
10	69	Meningiotheliomatous type/I	-	+
11	43	Meningioma/transitional type	-	-
12	69	Meningiotheliomatous type/I	-	-
13	49	Meningioma	Thyroid tumor (at the same age)	+
14	36	Meningioma/atypical	-	-
15	50	Metastatic carcinoma	Primary site: Lung	+
16	51	Metastatic brain	Primary site: Breast	+
17	53	Metastatic brain-breast origin	Primary site: Breast	+
18	39	Cerebellar medulloblastoma/IV	Micro-vascular proliferation	+

observed in patient 5 for C1 category (Figure 1).

The horizontal comparison presents diversity of ratio between the proteins; and the vertical comparison focuses on the intensity spectrum of PE, diversity, and the manner of any cascade alteration(s). Analysis of cells was based on the fluorescence intensity including 0: Lack of expression; 1: Low; 2: Medium; and 3: High expression for CCL2, EGF, and VEGF and the complementary markers (Figure 1).

The traditional direct/manual-based analysis guaranteed the adequate enumeration and detection of cells and heterogeneous pattern of PE, respectively, with more benefits including: (1) Analyzability of the quantitative and qualitative mode of PE; (2) Tracing the vasculature system; (3) Sensitivity of assay; (4) Possibility of multi-assay of the key markers and co-expression; and (5) Analysis of the multi-clonal populations and sub-population. So, this manner led to the quantitative- and qualitative-based classification of tumor cells in both tumor and CTCs. In addition, D1853N polymorphism was tested, which plays a predisposing role for probable occurrence of further exonic and/or intronic alteration(s) through different periods of patients' lives[21-23].

Preparative assessment

The extracted cells from 4 mL heparinized PB were stained with anti-CCL2 (monocyte chemoattractant protein-1) (eBioscience, San Diego, CA, United States), VEGF (Genature, Belgium), and EGF (Novus Biologicals, Littleton, CO, United States). This mixture was incubated at 4 °C. Staining was carried out by second layer antibodies, including fluorescein isothiocyanate for CCL2, R-phycoerythrin for VEGF, and phycoerythrin–indodicarbocyanine (Pe–Cy5) for EGF. Finally, by adding nuclear dye [4′,6-diamidino-2-phenylindole (DAPI)], the status of PE was assayed by the Leica EICA, DM RXA2-fluorescence microscope (Wetzlar, Germany). The CTCs were also assayed with cytokeratin 19 (KRT 19) (ScyTek, Logan, UT, United States), leukocyte common antigen [LCA cocktail (CD45) (BioCare Medical, Pacheco, CA, United States)], neuronal marker (NM) (ScyTek), and DAPI. Identification of brain CTCs was based on positive cells with DAPI/NM, and positive metastatic cells were confirmed

Proteins\ Tum ID	C0	C1	C2	C3	Ctot	V0	V1	V2	V3	Vtot	E0	E1	E2	E3	Etot
1	415.64	2.29	2.51	1.95	2.14	386.59	0.28	2.52	1.30	1.88	201.34	1.49	2.46	2.03	2.03
2	639.56	2.19	3.2	2.25	2.53	595.14	2.66	3.21	1.73	2.20	307.69	1.41	3.13	3.37	2.81
3	524.44	3.06	2.73	2.23	2.60	486.14	2.66	3.30	2.33	2.56	252.25	1.68	3.17	3.40	2.92
4	278.9	2.63	2	3.22	4.08	300.18	3.14	2.06	0.35	2.69	158.36	1.56	1.84	5.25	3.25
5	131.12	1.00	0.61	2.17	1.60	142.63	0.73	0.55	2.10	1.58	68.44	0.13	0.36	3.81	1.79
6	286.34	2.66	1.90	3.22	2.82	305.73	2.52	1.96	3.19	2.95	148.80	1.37	2.38	5.7	3.56
7	239.36	2.51	1.41	2.65	2.34	251.97	1.79	1.63	2.46	1.88	132.51	1.20	1.2	4.34	2.56
8	128.19	2.30	0.90	2.34	1.94	138.15	2.35	1.13	2.16	1.94	72.36	1.56	1.09	4.04	3.41
9	113.37	2.33	2.28	1.87	1.97	88.13	2.6	1.6	4.72	3.90	76.44	3.75	3.2	4.64	4.23
10	198.42	6.00	4.85	3.72	4.06	155.05	5.2	6.8	4.18	4.75	133.76	3	7.6	6.94	6.46
11	185.07	8	6	4.51	5	144.49	5.4	8.2	5.22	5.71	124.88	2	4.32	8.35	7.07

Proteins \ Tum-ID	C0	C1	C2	C3	Ctot	V0	V1	V2	V3	Vtot	E0	E1	E2	E3	Etot
12	678.	1.73	3.03	4.64	3.34	180.4	4.53	1.76	7.07	3.96	164.0	3.06	2.79	7.24	4.38
13	231.44	2.36	2.12	2.6	2.29	61	7.46	1.57	9.5	5.16	55.05	4	2.23	2.68	2.67
14	903.33	3.6	5.63	9.66	6.75	241.17	11.0	3.90	12.5	7.93	220.16	6.8	5.52	14.58	8.91
15	16.37	0.92	2	1.00	1.34	17.34	0.61	0.89	1.13	0.97	17.70	0.8	1.07	1.12	1.05
16	41.44	0.7	0.77	0.80	0.84	42.36	0.51	0.96	0.71	0.66	43.71	0.6	1.11	0.62	0.66
17	46.20	2	1.96	1.39	1.62	48.60	0.78	1.60	1.13	1.12	45.79	0.06	2.31	1.72	1.57
18	7.46	5.76	4.65	4.74	4.08	88.85	1.16	5.57	4.62	4.08	59.66	1.09	5.38	5.42	3.64

Figure 1 Ratio distribution of the cells lacking- and the classified positive-protein expression of C-C chemokine ligand 2, vascular endothelial growth factor, and epidermal growth factor between tumor cells in tumor sample to the circulated brain tumor cells. Patients 1-11: Are affected with meningioma. Patients 12-14: Are affected with meningioma, and patients 15-18 include three metastatic carcinomas (15: Brain/lung-origin; 16 and 17: Brain breast-origin) and one with cerebellar medulloblastoma. Provided horizontal and vertical indices are related to: C0, V0, and E0: Ratio related to lack of protein expression in C-C chemokine ligand 2 (CCL2), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF), respectively; C1, V1 and E1: Low expression of CCL2, VEGF, and EGF respectively; C2, V2, and E2: Medium protein expression of CCL2, VEGF, and EGF, respectively; C3, V3, and E3: High expression of CCL2, VEGF, and EGF, respectively; Ctot, Vtot2, and Etot2: Total ratio is related to the low plus medium, plus high protein expression of CCL2, VEGF, and EGF, respectively. C: C-C chemokine ligand 2; V: Vascular endothelial growth factor; E: Epidermal growth factor.

through DAPI$^+$/cytokeratin$^+$/CD45$^-$). The Manuel search was also performed to detect CTCs amongst the leukocytes. The CTCs were defined as nucleated cells by: (1) Expressing NM; and (2) Lacking CD45/positive KRT19. CD133 (Biolegend, San Diego, CA, United States), VEGF (Genature), and cyclin E (Zymed, San Francisco, CA, United States) were also assayed.

Ethics and consent declaration

The present study was approved and granted by the deputy of Research, TUMS (32208-30-04-95). The written consent declaration was obtained from the patients at Department of Neurosurgery, Shariati Hospital, TUMS.

Statistical analysis

By considering the personalized insight, the ratio test was applied between the CTCs and the T cells in tumor tissue and the vascular system.

RESULTS

The total analyzed cell population was ranged between 10794-92283 for T cells and between 117-2870 for CTCs. Characteristics of histopathologic and status of an ataxia-telangiectasia mutated polymorphism (D1853N) in 18 patients affected with brain tumors are provided (Table 1). The information in the table was provided by the surgeon and the patients.

Distribution of the intensity-based T/CTCs-ratio of PE for C, V, and E in the blood territory of patients 1-14 affected with meningioma (Figure 1) and in the patients 15-18 affected with malignant brain tumors, including one lung metastatic carcinoma, two metastatic carcinomas with breast origin, and one with CM are provided (Figure 1). The harmonic ratio of tumor cells lacking PE of C, V, and E to the CTCs in patients

with meningioma and brain cancer is illustrated in Figure 2A and B. The ratio of co-expression for tumor cells to the migrated tumor cells in the blood and the vascular system between proteins CCL2, VEGF, and EGF of the patients (Nos. 1-14) affected by meningioma is also provided (Figure 3A). The ratio of co-expression in tumor cells to the migrated tumor cells in the blood and the vascular system between these proteins of patients with brain cancer (patients 15-18) are presented (Figure 3B).

The harmonic manner of elevated or declined ratio for each protein is illustrated in a periodic chart (Figure 4). In this figure, the progressive intensity of proteins C, V, and E is traceable. Status of PE and co-expression of C, V, and E in CTCs of patients 1 and 9 (Figure 5) and in patient 4 (Figure 6) with meningioma are provided. Expression status of these proteins in meningioma tumors, vasculatures, and CTCs in patient 11 (Figure 7) and in patient 13 (Figure 8) are also available. In patient 15 with lung metastatic carcinoma, the status of PE and co-expression of CCL2/VEGF/EGF through migration of tumor cells to the blood territory is shown (Figure 9). The migrated vasculature into the BS, the CTCs in patient 15 (Figure 9A-G and in patient 17, and expression of these three proteins in the migrated vasculature into the BS are presented (Figure 10). The CTCs are shown in patient 18 with CM (Figure 11). Furthermore, the mode of PE of NM/CD133/VEGF in the CTCs of patient 1 with meningioma is presented (Figure 12). The PE of KRT19/CD45/cyclin E in patients 16 and 17 with metastatic brain tumor is provided (Figures 13 and 14, respectively).

DISCUSSION

Somatic/CTCs Ratio-based classification of PE

Programming of neoplasms is based on an evolutionary-mechanism that is not completely clarified in brain tumors. The T/CTCs ratio of cells lacking expression of C, V, and E indicate the highest value amongst all patients (Figure 1). The ratio of cells lacking PE, including C0, V0, and E0, reflects lower value in two patients with metastatic brain tumors (patients 16 and 17) and highlights the lowest value in CM-patient (*i.e.* more migrated tumor cells to the BS) (Figure 1). Furthermore, the declined or elevated values of T/B ratio play an important role in initiation and promotion of an evolutionary and developmental process through which transformation of the tumor cells lacking PE to the cells with positive PE, by gaining different intensity of expression will be possible. So, the sequential ratio is heterogeneous, varying within ranges of 113.37-903.33 in CCL2, 61-595.14 in VEGF, and 17.70-307.69 in EGF (Figure 1). Such pattern for the tumor cells is a challenging item in cancer initiation and progression. The question is whether such diversity has positive or negative impact on the invasiveness of tumor. An essential step in T progression is the most cooperative manner between relevant proteins, and a successful protein co-expression depends on the stage of disease, frequency of involved cells, and intensity of PE. Besides, systematic classification of the initiator factors would facilitate innovation of therapeutic channels. Distribution of ratios for C, V, and E is indicative of: (1) Diversity between different patients with the same tumor and between the categorized intensity-based ratios and total-based ratios, including total C, total V, and total E (Figure 1); and (2) The origin of evolution at the primary tumor, followed through blood with further behavior in the host organ (unpublished data). Besides, remarkable poor prognosis is related to the ratios less than 1. The AS is based on the intensity of expression that varied in different proteins and is provided as C1 (patient 5), V1 (patients 1 and 5), E1 (patient 5), C2 (in patients 5 and 8), V2 (patient 5), E2 (patient 5), and V3 (patient 4). An equal ratio is, exceptionally, observed in patient 5 for C1 category (Figure 1).

The harmonic- and non-harmonic status of the provided ratios for C/V/E is due to the different intensity-based classification through the course of evolution (Figure 2A and B). According to the co-expression images (Figure 9A and B), the migrated cells are, diversely, observed in the blood territory, ranging between 1.06-24.85 for the meningioma's and 0.08-0.92 for malignant tumors (Figure 3A and B). Co-expression including CCL2/VEGF, CCL2/EGF, EGF/VEGF, and CCL2/VEGF/EGF reveals the higher frequency of cells in T- than in blood-territory. Interestingly, only the ratio of PE in the vesicles, vasculature, and microvesicles is found to be higher in blood than in tumor, which may be the sign of poor prognosis for patients 5, 7, 8, 9, 13, 15, 16, 17, and 18 (Figure 3A and B). Convincingly, it is aimed that a complementary AS can provide more details on the nature and evolutionary process at tumor and blood levels for offering the road map of CTCs in brain tumors and other neoplasms. The key role of circulating micro-vesicles has been highlighted in leukemic cell-to-cell cross talk by

A

PS\ID	HL	RLH	LL
1	C/V/E		
2	C/V/E		
3	C/V/E		
4		C/V	
5		C/V	
6		C/E	
7		C/V/E	
8		C/V	
9		C/V	
10		C/V/E	
11		C/V/E	
12		V/E	
13			V/E
14	C/E		

B

PS\ID	HL	RLH	LL
15			C/V/E
16		V/E	
17		C/E	
18	V/E		

Figure 2 Ratio distribution of the cells lacking any protein expression of C-C chemokine ligand 2, vascular endothelial growth factor and epidermal growth factor between tumor cells in tumor sample to the circulated brain tumor cells in the blood. Patients 1-14: Are affected with meningioma and patients 15-18 include 3 metastatic carcinomas (15: Brain/lung-origin; 16 and 17: Brain/breast-origin) and 1 with cerebellar medulloblastoma (CM). A: Includes patients 1-14 affected with primary meningioma; B: Includes patients 15-18 affected with the metastatic carcinoma and CM. Harmonic ratio of tumor cells to the circulated brain tumor cells are marked as specific colored quadrants related to the intensity shared classification based between different proteins. PS: Protein status; HL: Highest level; RHL: Relatively high level; LL: Low level; ID: Patients' identification.

A

P.Exp.\ ID	CV	CE	EV	CVE	Ves	Vas	MV
1	1.65	1.90	1.81	2.15	3.14	2.5	1.52
2	1.79	2.72	2.21	2.28	6.52	4.62	2.78
3	2.57	3.07	2.36	2.90	2.85	3.12	3.79
4	3.08	4	2.87	2.85	3.28	1.27	1.23
5	1.77	2.23	1.60	1.76	0.4	3	1.21
6	2.95	3.66	2.91	3.26	1.16	2.1	1.56
7	2.20	3.72	2.29	2.47	2.36	19.4	0.22
8	1.84	2.61	2.19	2.01	0.92	0.45	1.05
9	2.17	2.89	2.17	1.63	1.2	1.12	0.4
10	3.6	4.24	2.91	2.41	8.3	7.25	4
11	4.91	5.06	3.63	2.47	6.1	4.62	4.55
12	5.03	7.03	4.45	3.87	5.57	6.15	5.95
13	3.57	5.45	3.45	2.29	3.25	0.8	4.45
14	9.32	12.78	9.70	7.81	68/0	3.05	24.85

B

P.Exp.\ ID	CV	CE	EV	CVE	Ves	Vas	MV
15	3.04	2.54	2.29	1.38	0.6	18.0	0.53
16	2.09	1.41	1.41	1.04	7.0	0.46	0.50
17	1.62	1.06	1.48	1.64	0.39	0.08	2.05
18	5.05	4.27	3.97	3.05	1.35	0.50	7.88

Figure 3 Ratio of co-expression of proteins between tumor cells in tumor region to circulating tumor cells and vasculature system for proteins C-C chemokine ligand 2, vascular endothelial growth factor, and epidermal growth factor in 18 patients with brain tumor. A: The ratio of co-expression between proteins C-C chemokine ligand 2 (CCL2), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) traced in tumor cells to the migrated tumor cells in the blood and the vascular system of patients with meningioma (pts. 1-14); B: The ratio of co-expression between proteins CCL2, VEGF, and EGF traced in tumor cells to the migrated tumor cells in the blood and the vascular system of patients with brain cancer (pts. 15-18). The highlighted quadrants with different colors indicate the harmonic evolutionary altered intensity of related proteins in common amongst different patients. Pr. Exp.: Protein expression; ID: Patients' identification; C: C-C chemokine ligand 2; V: Vascular endothelial growth factor; E: Epidermal growth factor; Ves: Vesicles; Vas: Vasculature; MV: Microvesicles (angiogenesis).

integrating the key proteins *in vitro*[21]. They have stated that the quantity of micro-vesicles can increase cell proliferation. In addition, our results highlight the quantitative analysis of involved proteins including VEGF in microvesicles of the tumor and CTCs with a persuasive impact on the clinical management. Interestingly, our results opposed the complete capability of BBB and highlight a complete availability of brain CTCs.

By considering degree of the PE-intensity, C, V, and E have medium status in common with ratio less than 1. Such pattern may provide poor prognosis for patient 5 with meningioma, micro-metastasis, and positive D1853N polymorphism. Furthermore, this patient had also minimum ratio of T/CTCs for low and medium intensity of VEGF and EGF in common, which is also a sign for poor prognosis. Two

PS\ID	Harmonic
1	V1>V2&E1>E2 & V2>V3 & E2>E3
2	C1>C2& V1>V2& E1>E2 & C2>C3 &V2>V3
3	C2>C3&V2>V3 & V1>V2 &E1>E2
4	C1>C2&V1>V2 & C2>C3&E2>E3
5	C1>C2&V1>V2& C2>C3&V2>V3&E2>E3
6	C1>C2&V1>V2& C2>C3&V2>V3&E2>E3
7	C1>C2&V1>V2 &C2>C3&V2>V3&E2>E3
8	C1>C2& V1>V2&E1>E2 & C2>C3&V2>V3&E2>E3
9	C1>C2& V1>V2&E1>E2 & V2>V3&E2>E3
10	C2>C3&V2>V3&E2>E3
11	C2>C3&V2>V3
12	V1>V2&E1>E2 & C2>C3&V2>V3&E2>E3
13	C1>C2& V1>V2&E1>E2 & C2>C3&V2>V3&E2>E3
14	C2>C3&V2>V3&E2>E3

PS\ID	Harmonic
15	C1>C2&V1>V2&E1>E2
16	C1>C2&V1>V2&E1>E2
17	C2>C3&V2>V3&E2>E3
18	V1>V2&E1>E2& C2>C3&E2>E3

Figure 4 The harmonic ratio of tumor cells with different degree of expression intensity for protein C-C chemokine ligand 2, vascular endothelial growth factor, and epidermal growth factor to the circulating tumor cells in patients with brain tumors. C-C chemokine ligand 2 (CCL2), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) are conjugated with fluorescein isothiocyanate, R-phycoerythrin, and phycoerythrin–indodicarbocyanine, respectively. The highlighted quadrants with different colors indicate the harmonic evolutionary altered intensity including increased and/or decreased ratio value of the related proteins in common between different patients or within different proteins. The altered ratio is based on the provided data in Figure 1; Numbers 1 (high), 2 (medium) and 3 (low) are reflective of expression intensity of proteins CCL, VEGF and EGF. C: C-C chemokine ligand 2; V: Vascular endothelial growth factor; E: Epidermal growth factor.

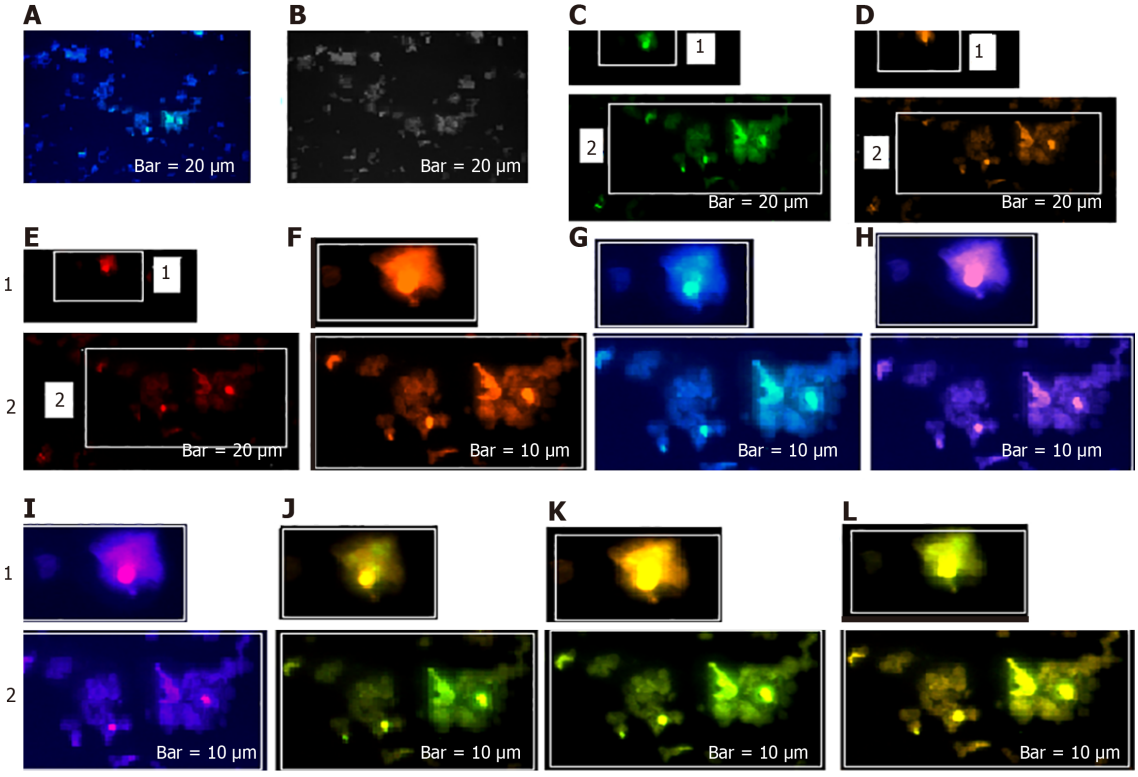


Figure 5 Mode of protein expression and co-expression of C-C chemokine ligand 2, vascular endothelial growth factor, and epidermal growth factor in circulating tumor cells of a patient with meningioma. C-C chemokine ligand 2 (CCL2), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) are conjugated with fluorescein isothiocyanate, R-phycoerythrin, and phycoerythrin–indodicarbocyanine, respectively, in patient number 1. A: Merged of 4',6-diamidino-2-phenylindole/CCL2; B: Phase contrast; C: Same cells with CCL2; D: Cells with VEGF; E: Cells with EGF; F: Merged/cropped cells with VEGF/EGF; G: Merged of 4',6-diamidino-2-phenylindole /CCL2; H: Merged cells with dai/VEGF; I: Merged cells with dai/EGF; J: Cropped/merged cells with CCL2/VEGF; K: Cropped/merged cells with CCL2/EGF; L: Cropped/merged cells with CCL2/VEGF/EGF.

patients with metastasis (15 and 16) are unique examples with poor prognosis by having < 1 ratio of cells with low PE in common; patient 16 also had different degree of PE for CCL2 and VEGF (Figure 1). Poor prognosis is related to the low expression of VEGF and EGF (T/B ratio 0.78 and 0.06 respectively) in patient 17 as a breast cancer (BC)-metastatic patient and positive/predisposing D1853N polymorphism. This

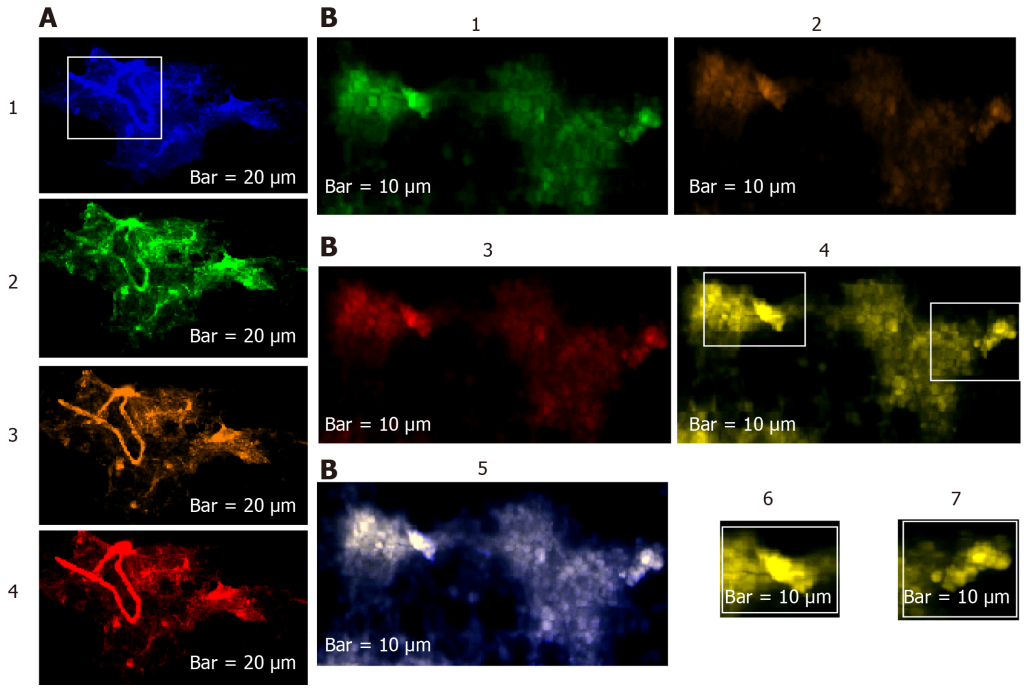


Figure 6 Status of protein expression of C-C chemokine ligand 2, vascular endothelial growth factor, and epidermal growth factor in the tumor and circulating tumor cells of patient 4 affected with meningioma. C-C chemokine ligand 2 (CCL2), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) are conjugated with fluorescein isothiocyanate, R-phycoerythrin, and phycoerythrin–indodicarbocyanine, respectively, in patient number 2. A1-4: 4',6-Diamidino-2-phenylindole, CCL2, VEGF, EGF; B1-5: CCL2, VEGF, EGF, co-expression of CCL2/VEGF/EGF, co-expression of 4',6-diamidino-2-phenylindole/CCL2/VEGF/EGF, Selected cropping area of image B4, is reflective of diverse co-expression between these three proteins from image B4.

polymorphism led to occurrence of further exonic and/or intronic alterations with negative impact on the clinical outcome[22-24].

The ratio of T/CTCs for lacking PE in C, V, and E may be harmonic and categorization of the ratios will have an influential and complementary role in the progression of tumor and clinical management (Figure 2A and B). By considering harmonic ratio of tumor cells lacking PE, seven patients (1-3, 7, 10, 11, and 15) reflect a remarkable triangle harmonic status of C/V/E, followed by dual harmonic status of C/V in patients 4, 5, 8, and 9; dual pattern of V/E in patients 12, 13, 17, and 18; and dual harmonic pattern of C/E in patients 13 and 17. These data reflect involvement of the triangle PE within diverse values in different patients. So, the cooperation between different proteins with diverse ratio may facilitate the initiation and promotion of the harmonic/cascade/evolutionary events as the elevated and/or decreased ratio as well.

By considering a novel categorization, different patterns of evolution, including dual-, triple-, and multi-, model are provided. The altered intensity (low: 1, medium: 2, high: 3) of expression leads to transform low-intensity to medium or high in C, V, and E proteins that include: (1) Dual pattern from V1 > V2 and from E1 > E2 in patients 1, 2, 9, 12, 13, and 18; (2) Dual model as V2 > V3 and E2 > E3 in patients 1 and 12; (3) Triple model as C1 > C2 and V1 > V2 and E1 > E2 alterations in patients 2, 9, 13, 15, and 16; triple pattern formation as C2 > C3, V2 > V3 and E2 > E3 in patients 10, 12, 13, 14 and 17; and (4) Multi pattern development including C1 > C2, V1 > V2, C2 > C3, V2 > V3, and E2 > E3 in patient 8 who harbors the most complicated evolutionary model in which all the initiative events are accompanied by complimentary functional incidents. So, the model is initiated simply by the occurrence of dual-evolution and is followed by further diverse development of triple-events. Conclusively, to form the completed model, diverse dual and triple patterns are required to be formed, and the priority of initial formation could occur from the low expression with further development to medium- and high-intensity of expression (Figure 4A and B). These evolutionary models in our patients reflect the occurrence of cascade events with common origin. The provided model indicates the cross-talk between initiative and the complementary channels to form an evolutionary insight in cancer. Such pattern could occur in all types of neoplastic tumors with further evolution through the metastatic process (unpublished data). It is essential to emphasize that such models vary within different proteins and patients. Therefore, these patterns are rather personalized with specific and multi-combinational patterns.

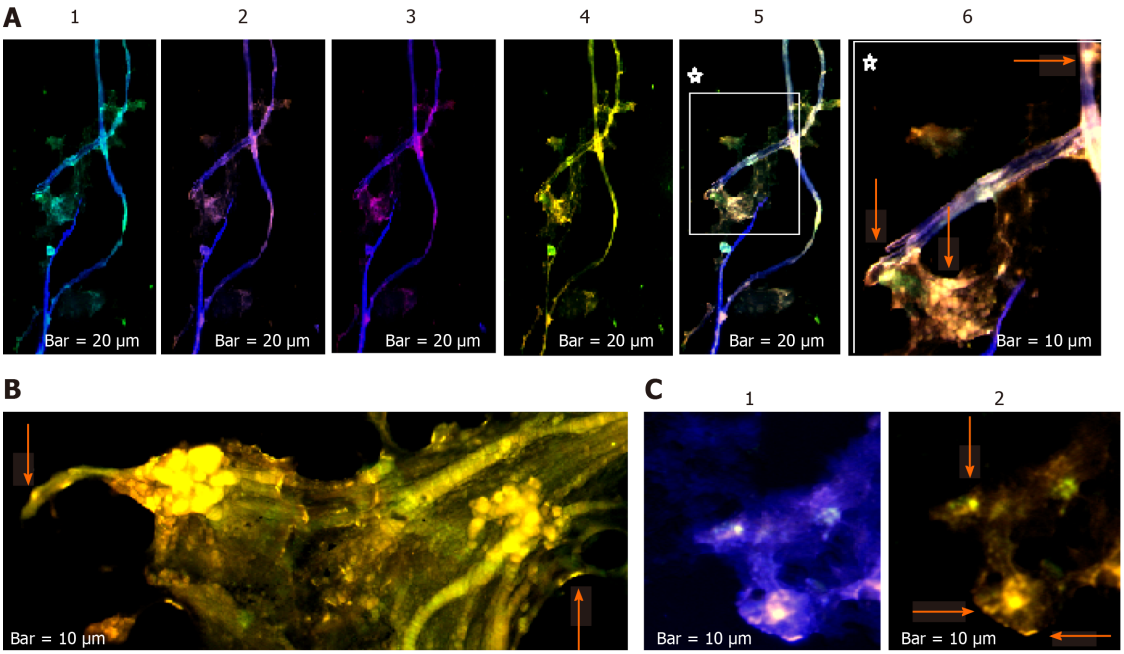


Figure 7 Protein expression status of C-C chemokine ligand 2/vascular endothelial growth factor/epidermal growth factor of two cell population from tumor sample and circulating tumor cells in patient 11 affected with meningioma. C-C chemokine ligand 2 (CCL2), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) are conjugated with fluorescein isothiocyanate, R-phycoerythrin, and phycoerythrin–indodicarbocyanine, respectively, in patient number 3. A: Vascular images accompanied by the partial tumor section [1: Merged of 4',6-diamidino-2-phenylindole (DAPI)/CCL2; 2: Merged of DAPI/VEGF; 3: Merged of DAPI/ EGF; 4: Co-expression of CCL2/EGF/VEGF; 5: Co-expression of DAPI/CCL2/VEGF/EGF; 6: A cropped section of 5]; B: Image of a tumor section reflecting of the Co-expression of CCL2/VEGF/EGF; C [1:DAPI/CCL2/VEGF/EGF; 2:CCL2/VEGF/EGF]: Illustrates the CTCs in the blood sample of the same patient. The arrows are indicative of expression status in tumor cells, in vasculature, and the microvesicles.

Status of PE

In patients 5 and 8, the higher PE in CTCs may be indicative of the occurrence of an evolution in favor of the metastatic process (Figure 5). Interestingly, the manner of PE was more diverse between the cells in tumor than in the migrated cells into the vascular system within the tumor region (Figure 6A) and also in the CTCs within the blood territory in patient 4 with meningioma (Figure 6B). Status of PE in CCL2/VEGF/EGF of the two cell populations from tumor sample of patient 11 and CTCs presents the journey from tumor to the destinations, including the vascular- and BS-territory (Figure 7). In spite of the lowest expression of EGF/VEGF (Figure 7C and D), higher expression with CCL2 and the co-expression within CTCs is noticeable in patient 13 (Figure 8E and G). Migrated tumor cells within tumor territory to the BS are shown by arrows (Figure 9C-H) in which the co-expression of CCL2/VEG and CCL2/VEGF/EGF is more remarkable than in CCL2/EGF (Figure 9F-H). Regarding patient 17, more cells conjugated with CCL2 are observable than with VEGF and EGF (Figure 10B-D). Images C, D, and G reflected an observable expression in a single CTC with proteins VEGF and EGF but not with CCL2 in image B. In contrast, the mode of expression is diverse within the vasculature territory. There was a diverse co-expression pattern indicating more harmonic interaction between CCL2/VEGF than CCL2/EGF. However, co-expression between these proteins was remarkable either in CTCs or within the vasculature territory (Figure 10). In patient 18 with CM, co-expression between CCL2/VEGF/EGF is observable in CTCs (Figure 11A), but there is only a single CTC with high expression of CCL2 (Figure 11B). In contrast, high expression of VEGF and EGF, with slight difference between these two proteins, is observable (Figure 11C and D).

For tracing the tissue origin of CTCs, PE of NM is accompanied by stem cell marker CD133 and VEGF in patient 1 (Figure 12), presenting the cluster of cells with higher expression of NM (Figure 12B) and few cells with lower expression of CD133 and VEGF (Figure 12D and E). Interestingly, remarkable co-expression between NM/CD133/VEGF in CTCs may be indicative of a warning message for probable invasiveness of the tumor. The presence of CTCs was confirmed by the mode of cytokeratin-19^{+}/CD45^{-} in patients 16 and 17 (Figures 13 and 14).

The minimal residual disease was traced by CTCs in the blood of BC patients[25]. They have reported a poorer outcome by detecting ≥ 5 CTCs/7.5 mL of PB by

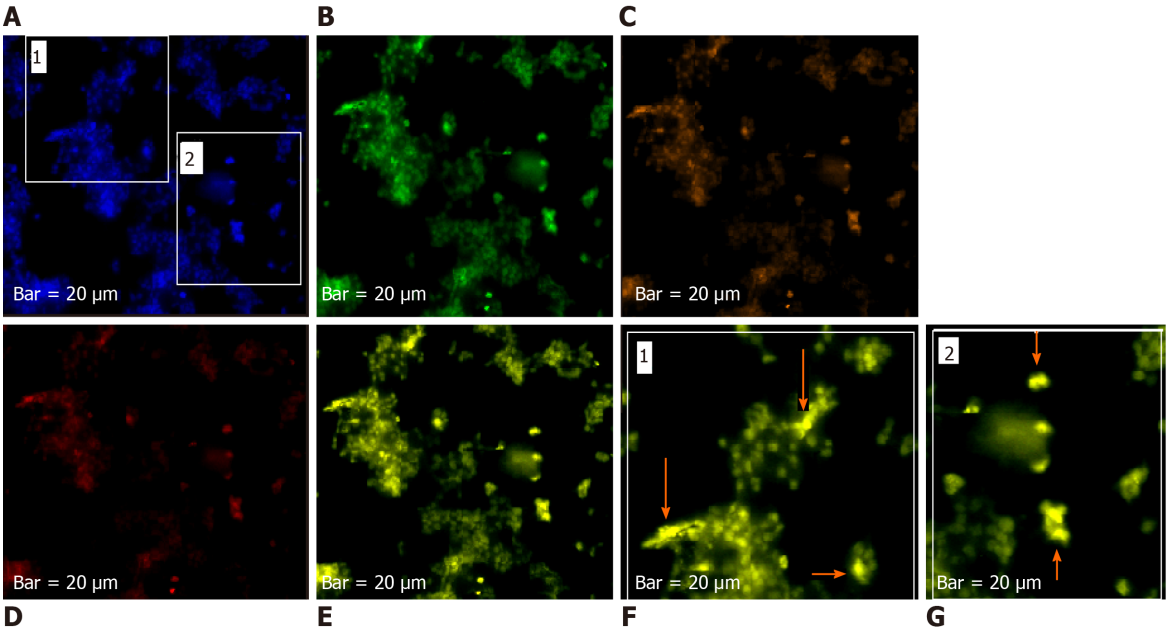


Figure 8 Mode of protein expression and co-expression of C-C chemokine ligand 2, vascular endothelial growth factor, and epidermal growth factor of the circulating tumor cells of patient 13 with meningioma. C-C chemokine ligand 2 (CCL2), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) are conjugated with fluorescein isothiocyanate, R-phycoerythrin, and phycoerythrin–indodicarbocyanine, respectively. A: Leukocytes and circulating tumor cells (CTCs) with 4',6-diamidino-2-phenylindole; B: Image of the same cells with CCL2; C: Image of the same cells with VEGF; D: Image of the same cells with EGF; E: Co-expression of the CCL2/VEGF/EGF in the same cells; F and G: Co-expression of the CCL2/VEGF/EGF in the selected cropping cells (1 and 2) from A. The arrows are reflective of high protein co-expression within CTCs according the mode of expression in images b-d in the EGF; and the VEGF reflects the lowest expression.

automated cell Search® system. Furthermore, presence of ≥ 1 CTC/7.5 mL pre- and post-chemotherapy has led to distant metastasis[26]. They also highlighted that although CTC was correlated with circulating VEGF, there was no association with status of EGF. So they have proposed that the CTCs may be a remarkable predicting response to the angiogenic inhibitors. CTCs are known as epithelial tumor cells, traceable in the BS of solid tumors at the molecular level. CTCs have also been considered in research and clinical management since 1990. But, no categorized cell-based diagnostic strategy as the translational research in cancer has been provided yet. There are the notable challenges on tracing of CTCs, so an alphabetic guideline is required for this topic. Therefore, we aimed to explore the scenario of the behavior of CTCs in brain tumor/blood samples to provide the personalized, hypothetical-based agenda in the brain neoplasms that can be applied in other types of neoplastic disorders; and also as a follow-up approach in the patients and their relatives who are at risk.

The prognostic potential of CTCs and the clinical significances in different tumors and a few examples on BC-brain metastasis are reviewed[27]. They have delivered the application of "Liquid biopsy" at mRNA level. The machinery of CTCs with focus on genomes and transcriptomes is also reviewed[28]. It was also reported that the presence of CTCs plays an independent role in prognosis of BC with brain or bone metastasis[29]. In spite of the presence of only ≥ 5 CTCs, they found a correlation between the undetectable CTCs and occurrence of metastasis in brain but not in bone. So, they concluded that undetected CTCs might be due to an incomplete tracing of CTCs by automated Cell Search system and possibly related to the "epithelial-mesenchymal transition. The first reason is cleared by the enumeration strategy in our present data. However, our data indicate that BBB may have very limited restriction on the tumor cell migration into the BS (Figure 15).

CONCLUSION

Heterogeneity/diversity may be an explanation for the Natural Rescue Mechanism through which the severity of disease may be minimized in brain tumors and hopefully in other neoplasms.

Adequate informative data on CTCs is a guideline for management of metastatic

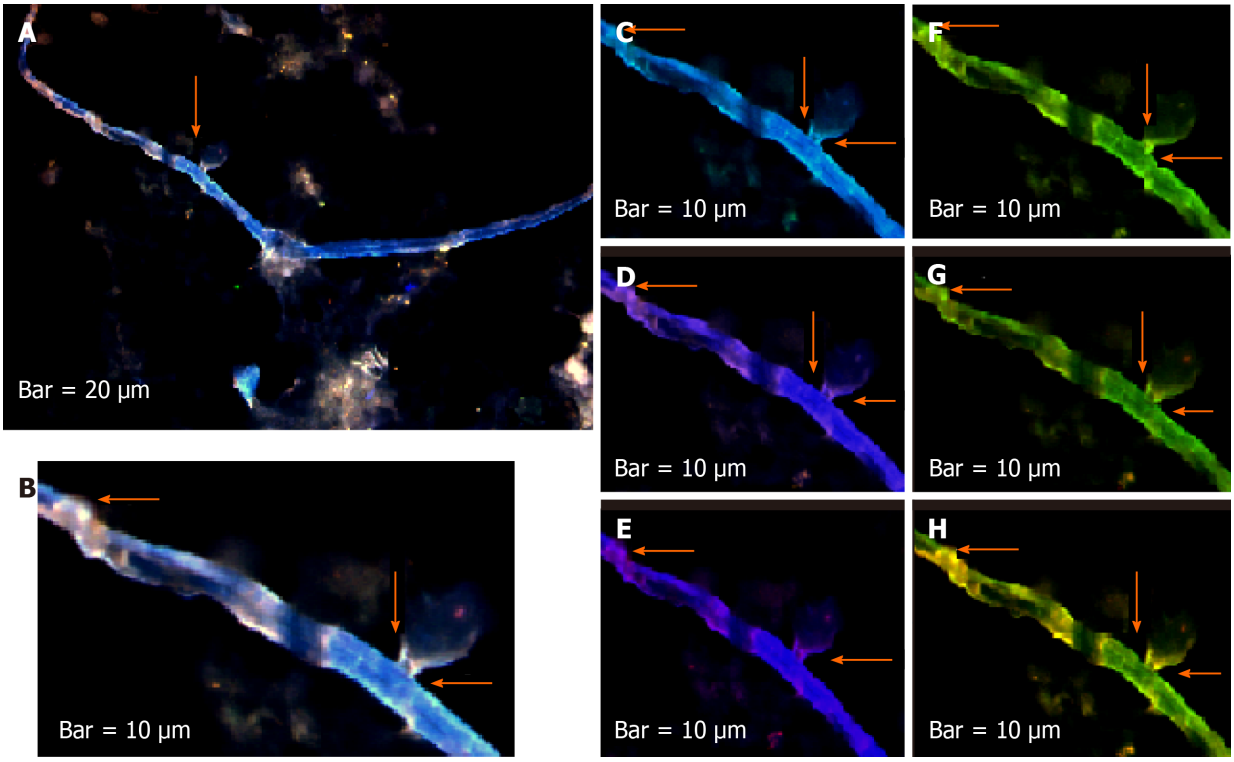


Figure 9 Status of protein expression and co-expression of C-C chemokine ligand 2/vascular endothelial growth factor/epidermal growth factor through migration of circulating tumor cells within tumor territory to the blood stream in a patient with the lung metastatic carcinoma. C-C chemokine ligand 2 (CCL2), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) are conjugated with fluorescein isothiocyanate; R-phycoerythrin, and phycoerythrin–indodicarbocyanine, respectively, in patient 15. A: Merged of 4',6-diamidino-2-phenylindole (DAPI)/CCL2/EGF/VEGF; B: Cropped section of image a; C: Merged of DAPI/CCL2; D: Merged of DAPI/VEGF; E: Merged of DAPI/EGF; F: Co-expression of CCL2/Veg; G: Co-expression of CCL2/EGF; H: Co-expression of CCL2/VEGF/EGF. Merged of DAPI/CCL2/VEGF/EGF is reflective of co-expression between three proteins; B, F-H: Reflect co-expression of CCL2/VEGF/EGF according to the selected of cropped section from images a (arrow on the top/left). The arrows on the left side of images are referred to the migration of tumor cells from tumor to the blood stream destination through a bubble like channel.

process. Regarding the application of anti-VEGF in brain tumors, the multi-disciplinary insight in the therapeutic strategy is also required. Interestingly, application of anti-CCL2 in tumorigenic models has led to weakening the metastasis and improving the survival period[30]. So, adequate enumeration of CTCs, formulated predictive information with a personalized-based strategy for different types of cancers, will be possible. As questions: Whether the high number of CTCs is reflective of (1) poor developmental course of neoplasms; and (2) poor survival status or recurrence? Or relapse? And does low enumeration of CTCs lead to the false negative diagnosis?

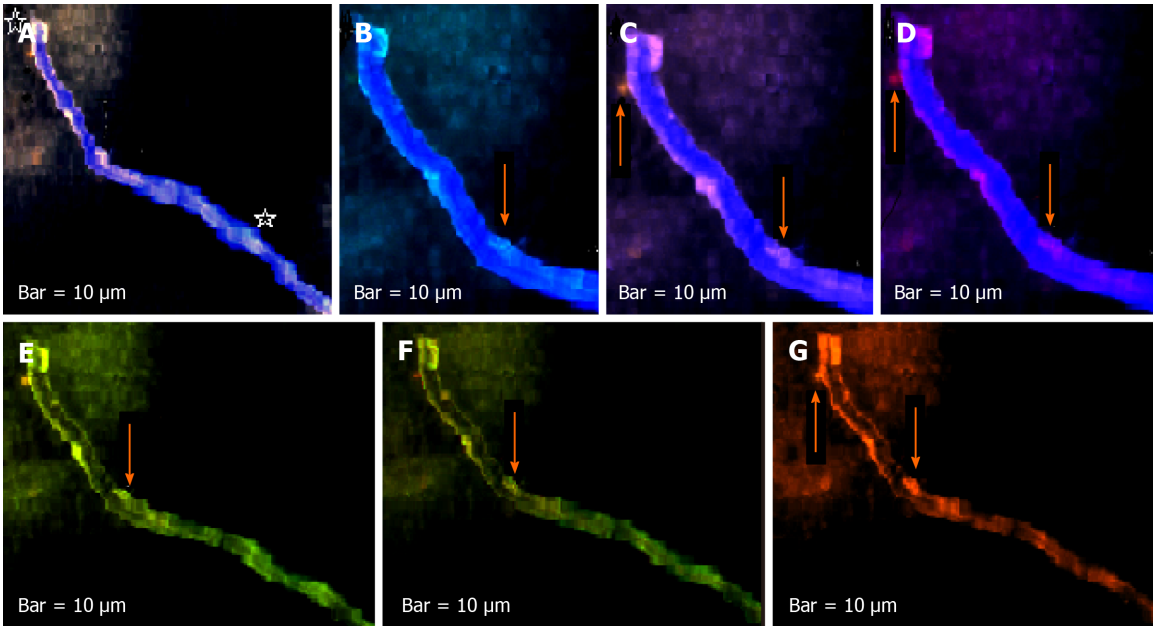


Figure 10 Expression mode of C-C chemokine ligand 2, vascular endothelial growth factor and epidermal growth factor in the migrated vasculature into the blood stream in a patient affected with metastatic brain tumor with breast origin. C-C chemokine ligand 2 (CCL2), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) are conjugated with fluorescein isothiocyanate, R-phycoerythrin, and phycoerythrin–indodicarbocyanine, respectively in patient 17. A: Merged of 4',6-diamidino-2-phenylindole (DAPI)/CCL2/VEG/EGF; B, C and D: Partially cropped section between two asterisk of image A and present the merged of DAPI/CCL2, DAPI/VEGF and DAPI/EGF respectively; B and C: Indicates the involvement of traceable tumor cells with higher expression than EGF within a migrated vasculature to the blood stream; E, F and G: Present co-expression between CCL2/Veg, CCL2/EGF and EGF/VEGF respectively, there is more harmony between CCL2/Veg and CCL2/Veg.

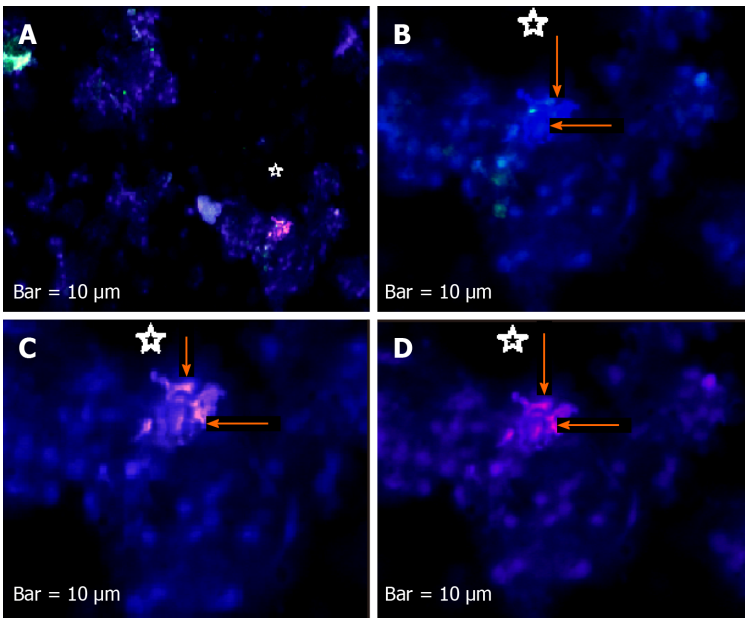


Figure 11 Expression mode of proteins of C-C chemokine ligand 2, vascular endothelial growth factor, and epidermal growth factor in circulating tumor cells from a tumor of patient affected with cerebellar medulloblastoma. A: Merged image of cells conjugated with 4',6-diamidino-2-phenylindole (DAPI)/C-C chemokine ligand 2 (CCL2)/vascular endothelial growth factor (VEGF)/epidermal growth factor (EGF) is reflective of remarkable co-expression between VEGF/EGF proteins; B: Merged cells with DAPI/CCL2 indicating the absence of expression in the majority of cells; C: Merged cells with DAPI/VEGF with high expression; D: Merged cells with DAPI/EGF reflect lower expression than VEGF. Asterisks are referred to the cluster of CTCs.

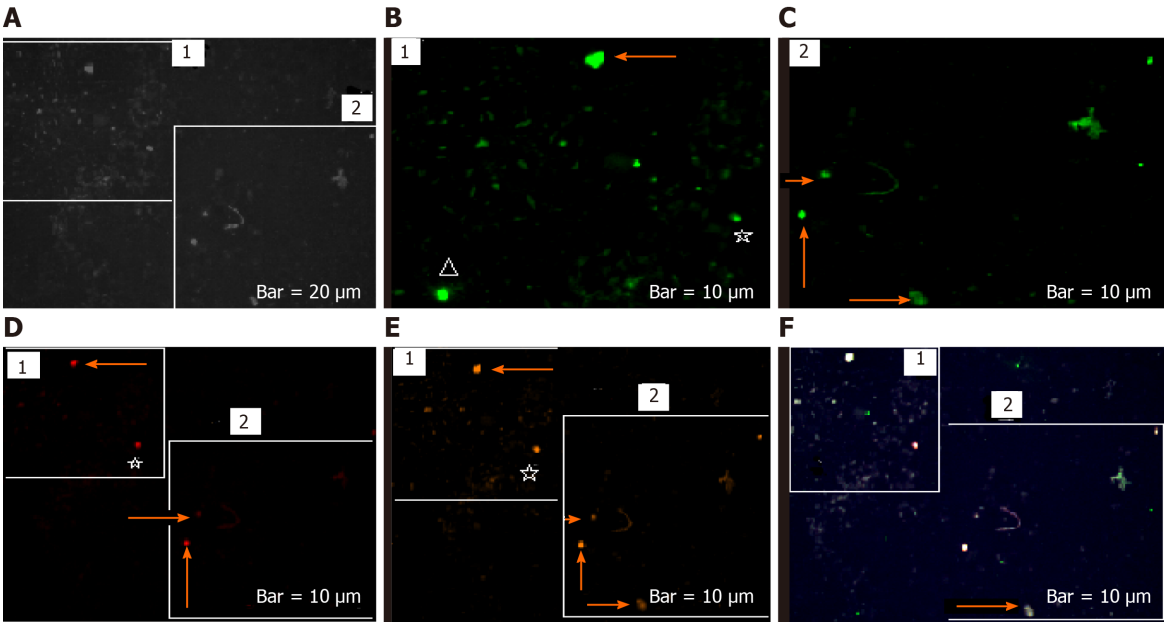


Figure 12 Status of protein expression of neuronal marker, CD133 and vascular endothelial growth factor in the brain circulating tumor cells of patient 1 affected with meningioma. A: Circulating tumor cells (CTCs) with phase contrast; B and C: Present CTCs with Ne conjugated with fluorescein isothiocyanate; D: Present status of vascular endothelial growth factor (VEGF), conjugated with phycoerythrin–indodicarbocyanine; E: Reflect status of CD133, conjugated with R-phycoerythrin; F: Present the merged image of 4',6-diamidino-2-phenylindole/Ne/VEGF/CD133, reflective of a remarkable co-expression between protein neuronal markers CD133 and VEGF. Arrows on the top of images B, D1, and E1 reflect more involved cells with high-, very less involved cells with much lower-, and less involved cells with high-expression for Ne, VEGF, and CD133, respectively. The asterisks on the down/right of B, B and E illustrate almost same intensity of expression, but with more CTCs involved in image B for Ne, VEGF, and CD133, respectively. A triangle symbol is reflective of a remarkable high expression for neuronal marker in CTCs. Arrows on the below/left-down of images C2, D2, and E2 present higher expression for Ne than VEGF and CD133. Arrows on the bellow/left-up of images C2, D2, and E2 illustrate low expression for Ne and CD133, and lack of expression for VEGF. The arrows down the images of C2, D2, and E2 illustrate the heterogenic (low and lack) of expression for Ne and CD133 and lack of expression for VEGF.

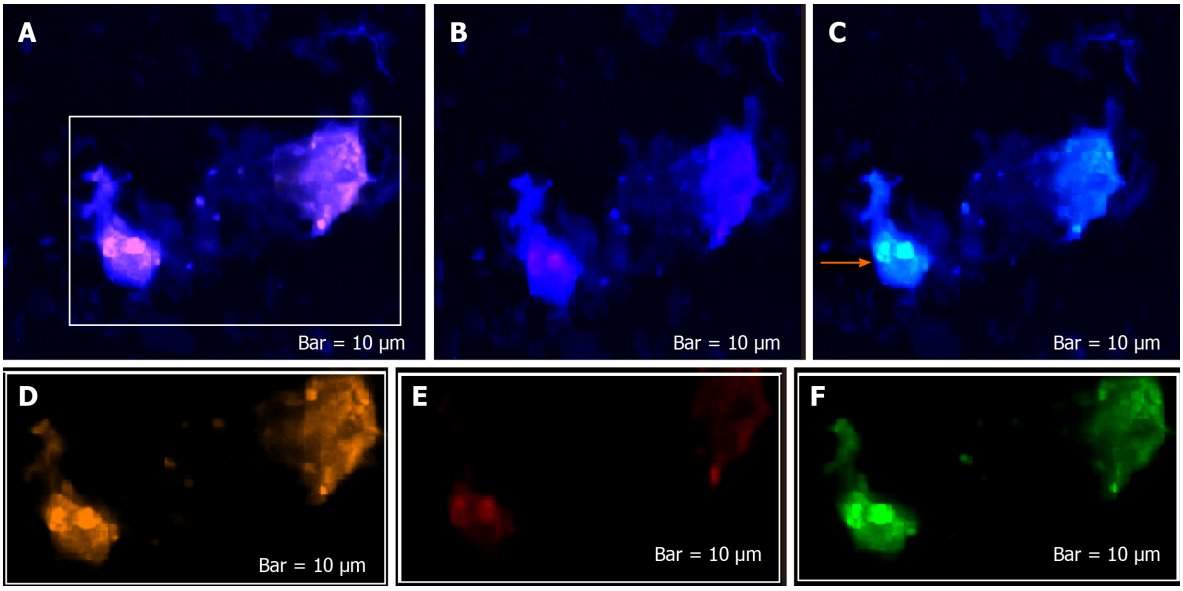


Figure 13 Protein expression of cytokeratin-19, CD45, and cyclin E in circulating tumor cells of a breast-brain metastatic patient. Circulating tumor cells (CTCs) in patient 17 with: A: DAPI (4',6-diamidino-2-phenylindole)/cytokeratin-19 (CK-19) [highest/positive protein expression (PE)]; B: DAPI/CD45 (negative PE); C: DAPI/cyclin E (positive PE: Partial at left clone of cells); D, E and F: Conjugated cells with CK-19, CD45, and cyclin E, respectively. Harmonic PE between CK-19 and cyclin E in the clone of CTCs at left side of image in patient 17. Arrows indicate the diverse expression of three proteins.

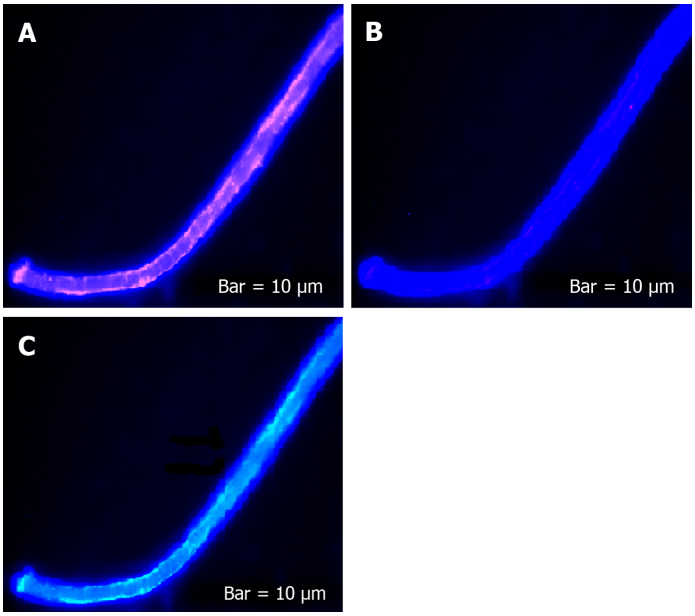


Figure 14 Protein expression of cytokeratin-19, CD45, and cyclin E in circulating tumor cells of a vasculature image of a breast-brain metastatic patient. Cytokeratin-19 (CK-19), CD45, and cyclin E are conjugated with phycoerythrin-indodicarbocyanine, R-phycoerythrin, and fluorescein isothiocyanate, respectively. A: 4',6-Diamidino-2-phenylindole (DAPI)/CK-19 [highest/positive protein expression (PE)]; B: DAPI/CD45 (negative PE); C: DAPI/cyclin E (positive PE: Partial at left clone of cells). Harmonic PE between CK-19 and cyclin E, arrows indicate lack of cyclin E expression in the middle of vasculature in patient 17.

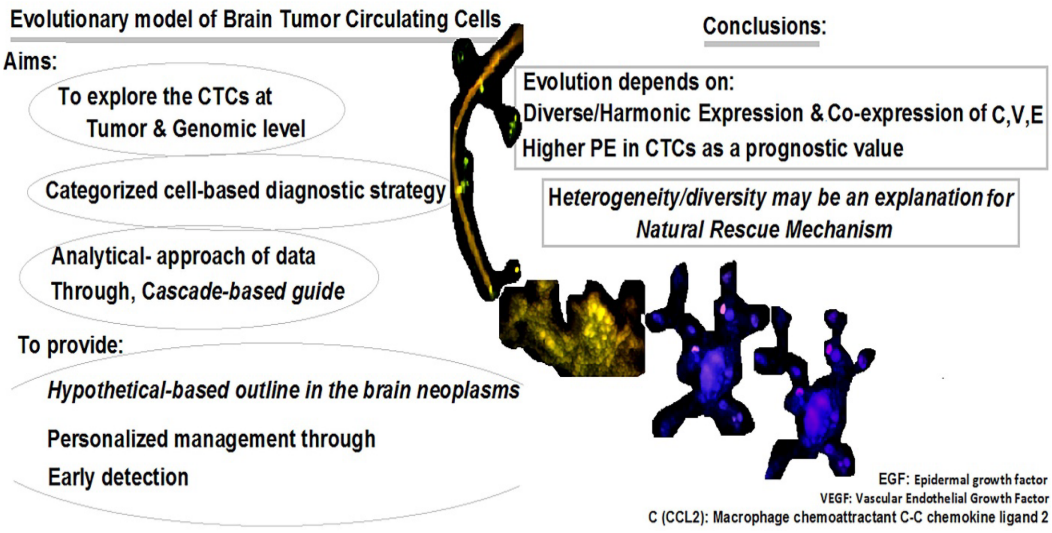


Figure 15 Manuscript at a glance: Evolutionary model of brain tumor circulating cells. CTCs: Circulating tumor cells; PE: Protein expression.

ARTICLE HIGHLIGHTS

Research background

Circulating tumor cells (CTCs) are the most applicable strategy in preventive oncology. The most puzzling items include the lack of: (1) The basic information on the migrated cells into the tumor and into the blood stream; (2) The categorized data for the personalized strategy of CTCs; (3) Information on the application of CTCs for the patients affected with brain tumors as early as possible; and (4) The data on the early detection of the brain tumors in the probands affected with brain tumor and the relatives through their pedigree. Hence, it was aimed to explore the developmental picture of CTC behavior in tumor (T) and blood in the brain neoplasms.

Research motivation

The key target to explore CTCs was revealed to be early detection; therefore, availability of adequate data on the pedigree of proband affected either with brain tumor or any other neoplastic disorder (s) will facilitate application of the preventive strategy for the probands' relatives who are at risk of being affected with the neoplastic disorders.

Research objectives

In the cancer-prone families: The target objectives, include: (1) The family history of any neoplastic disorders in the proband's pedigree and age of the onset for the target individuals; (2) The key information for each individual affected with neoplastic disorder including cancer; (3) Tracing and selecting the target relatives of the probands; and (4) Performing the CTC test for the selected individuals.

In the family of the probands without any history of the neoplastic disorders: In case of the influential micro-and/or macro-environmental hazards, the target offspring of the proband through the further generations may be selected for the performance of CTCs.

Research methods

The novelty of this research is relied on the simple Manuel analysis, which provides an adequate single cell screening process. Based on this strategy, diverse pattern of intensity and categorization of different target proteins will be facilitated.

Research results

The course of evolution and possibility of metastatic event rely on the higher protein expression in CTCs. Such a finding is indicative of prognostic value. Besides, diversity in protein expression of the migrated cells into the both tumor and blood stream and diverse pattern of protein expression in both tissues are indicative of the occurrence of evolution. Besides, the harmonic co-expression between C-C chemokine ligand 2/epidermal growth factor and C-C chemokine ligand 2/vascular endothelial growth factor can facilitate the tumor progression including the metastatic event.

Research conclusions

There is a diverse pattern of protein expression of the migrated cells at different territories including tumor and the bloodstream by performing CTCs by Manuel analytical strategy to unmask heterogeneity of the protein expression.

Research perspectives

The direction of further research includes providing: (1) The educational/informative packages on the early detection by CTCs to the cancer clinics or the related hospitals with the available preventive cancer section; and (2) Cancer Genetic counselling to the referral cancer patients for performing an early detection of probable metastasis by CTCs test.

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SYSTEMATIC REVIEWS

Adaptation of international coronavirus disease 2019 and breast cancer guidelines to local context

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Abstract

BACKGROUND

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (a novel coronavirus), which was first identified amid an outbreak of respiratory illness cases in Wuhan, China and declared a global health emergency, is currently considered an additional challenge in the management of patients with breast cancer (BC). Cancer patients are more vulnerable to becoming infected with severe acute respiratory syndrome coronavirus 2 and are more likely to suffer additional complications that can increase mortality. Identifying those BC patients who require more urgent therapy than others in the current situation is essential. These recommendations are based on and have been adapted from those similarly published by

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international scientific societies for BC management. They are divided mainly by clinical stage (early, advanced), subtype [luminal, human epidermal growth factor receptor 2 (HER2), triple-negative], or type of medical treatment and setting (neoadjuvant, adjuvant, metastatic). Recommendations for HER2 and triple-negative subtypes are similar, whereas in luminal subtype there are various options of management. The objective is to adapt guidelines to local context through relevant decision-makers, avoiding duplication of efforts and optimizing use or resources. We hope that these recommendations will help medical oncologists provide the best quality care to BC patients during the COVID-19 pandemic with information tailored to our healthcare system.

AIM
To establish and adapt recommendations from those published by international scientific societies for BC management.

METHODS
The Peruvian Society of Medical Oncology developed a consensus and propose here a manuscript with recommendations for oncological medical treatment of BC during the COVID-19 pandemic. The Peruvian Society of Medical Oncology invited a panel of experts and opinion leaders on BC working in major health care systems around Peru. Panel experts selected three international clinical practice guidelines (National Comprehensive Cancer Network, European Society for Medical Oncology, Spanish Foundation Research Group in Breast Cancer), considering that these are more representative in COVID-19 management. Also, the panel agreed to include at least one European and American clinical practice guideline.

RESULTS
Recommendations about BC management during the COVID-19 pandemic were divided mainly by clinical stage (early, advanced), subtype (luminal, HER2, triple-negative), or type of medical treatment and setting (neoadjuvant, adjuvant, metastatic). Recommendations for HER2 and triple-negative subtypes were similar between clinical practice guidelines, whereas in luminal subtype there were various options of management. One hundred twelve recommendations were reviewed, adapted, and voted. A consensus was made in order to provide best decisions of management, avoid duplication of efforts, and optimize medical resources, considering health care system reality. These recommendations are not intended to replace clinical judgment.

CONCLUSION
Most of recommendations are similar, mainly in high-risk subtypes (HER2, triple-negative). Certain societies adapt them to deal with different situations involving the best decision in the management of BC patients.

Key Words: Breast cancer; COVID-19; Guidelines; Recommendations; Oncology; Medical treatment

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Core Tip: This manuscript includes some recommendations about oncological medical treatment of breast cancer in coronavirus disease 2019 pandemic that were selected from three international clinical practice guidelines. These were reviewed and adapted to local context by a panel of experts from Peru invited by the Peruvian Society of Medical Oncology using levels of prioritization. Consensus was made, including a vote, in order to provide best decisions of management, avoid duplication of efforts and optimize medical resources, considering health care system reality. These recommendations are not intended to replace clinical judgment.

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INTRODUCTION

The rapid escalation of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to a pandemic has required a fast mechanism of prioritization of health services globally, to identify and select correctly actions that assure the best management of the breast cancer (BC) patient. The risk of worse outcomes needs to be weighed against the risk of patients and health workers to SARS-CoV-2 infection[1,2].

The need for priority mechanisms is mandatory since there are more restrictions, especially in systems with impaired availability or accessibility (including limited human resources and lack of access to medicines). The prioritization is established according to clinical practice guidelines, which are based on the highest-level available evidence[3-5].

MATERIALS AND METHODS

The Peruvian Society of Medical Oncology (SPOM, Lima-Peru) developed a consensus and proposed a manuscript with recommendations for oncological medical treatment of BC during the coronavirus disease 2019 (COVID-19) pandemic. SPOM invited a panel of experts and opinion leaders on BC working in major health care systems around Peru (nine in total). Panel experts selected three international clinical practice guidelines (National Comprehensive Cancer Network, European Society for Medical Oncology, Spanish Foundation Research Group in Breast Cancer), considering that these are more representative in the COVID-19 management. Also, the panel agreed to include at least one European and American clinical practice guideline. Previously, in an attempt to reduce debate and discuss controversial questions, panelists *via* teleconference had reviewed and exchanged recommendations.

By vote, panel of experts defined and adapted three levels of prioritization from European Society for Medical Oncology: High, medium and low priority; to support the best decision of care in oncological medical treatment, adapting treatment recommendations to the local context.

The consensus (Friday, June 19, 2020) was held in Lima, Peru *via* teleconference. A moderator chaired the panel discussion and the voting. One hundred twelve recommendations were reviewed, adapted, and voted. Panelists were asked to cast their vote using three possible answers: Yes, no, abstain. "Abstain" was used in cases of insufficient data, lack of expertise on the issue in question, or panelist's conflict of interest. A percentage greater than 70% was considered a unanimous decision. Furthermore, some additional comments were added on each topic under discussion.

RESULTS

General recommendations for the management of BC during the COVID-19 pandemic

During the pandemic, it is recommended to choose regimens and therapeutic sequences that are the most consistent with the current situation to reduce the risk of SARS-CoV-2 infection in patients and health workers, without compromising the prognosis of patients (Yes: 100%, No: 0%). In addition, there should be a differentiated triage for COVID-19 in all cancer centers before entrance, where patients and family members are questioned about their symptoms, history contacts, as wells as a record of body temperature for fever. In case of suspected COVID-19 infection, patients and/or companions should not have contact with other patients and/or health workers and should be managed according to protocols of each institution (Yes: 100%, No: 0%).

It is important to leave a medical register (informed consent) specifying explicitly the discussion with the patient of risk/benefits of treatments in the context of a pandemic as well as therapeutic decisions and alternatives available (Yes: 78%, No:

22%). In addition, individualizing the need for blood transfusions when strictly necessary (Yes: 100%, No: 0%) (Table 1).

Priorities of outpatient visits for BC during the COVID-19 pandemic

Events that require immediate in-person visit or evaluation and/or treatment (high priority) are, for example, unstable postsurgical patients (hematoma, infection, bleeding) (Yes: 100%, No: 0%) and oncological emergencies (febrile neutropenia, uncontrolled pain, symptomatic brain metastases) (Yes: 100%, No: 0%). Other conditions to evaluate in-person are BC diagnosis during pregnancy (Yes: 100%, No: 0%) and *de novo* locally advanced BC with aggressive phenotypes [human epidermal growth factor receptor 2 (HER2) positive, triple-negative] (Yes: 78%, No: 22%).

De novo invasive BC (Yes: 89%, No: 11%), intercurrences on-treatment patients (new signs/symptoms, abnormal findings during the physical examination, adverse events) (Yes: 100%, No: 0%), patients on active intravenous chemotherapy (Yes: 78%, No: 22%), and clinically stable routine postsurgical patients are examples of medium priorities (Table 2).

Priorities for telemedicine

Recommendations about telemedicine are generally classified as medium or low priority. The majority of patients could be evaluated remotely *via* telemedicine (if feasible) (Yes: 100%, No: 0%). In-person visits can be converted evaluating the risks of viral transmission to patients and health workers. Examples of patients with BC who can be evaluated with telemedicine are patients completing neoadjuvant chemotherapy and waiting for surgery (Yes: 89%, No: 11%), patients eligible for radiotherapy (first visit) (Yes: 89%, No: 11%), and patients receiving oral chemotherapy or endocrine therapy + targeted therapy (Yes: 78%, No: 22%).

Low priority patients are those for whom certain treatments or interventions can be deferred until pandemic is over; for example, routine evaluation in patients who are in periodic controls (observation) or endocrine therapy (Yes: 78%, No: 22%) and survivorship follow-up (Yes: 100%, No: 0%) (Table 3).

Priorities for breast disease: Diagnostics and imaging

As a high priority, clinical diagnosis of a mass or lump in the breast (with auto examination) or another sign with high suspicion of malignancy should be immediately evaluated (Yes: 100%, No: 0%). Similarly, patient should be evaluated when there is clinical evidence of relapsed locoregional disease (Yes: 100%, No: 0%). There are urgent situations that require imaging (Yes: 100%, No: 0%). It is recommended to perform additional images upon abnormal mammogram results (*e.g.*, Breast Imaging Reporting and Database System score 5) or suspected BC signs/symptoms (Yes: 89%, Abst: 11%) and for patients experiencing relapsed BC (Yes: 100%, No: 0%). Pathologic evaluation (cytopathology or histopathology) for abnormal mammograms or symptoms in the breast or symptomatic metastatic relapse is considered high priority (Yes: 100%, No: 0%).

Examples of medium priority in imaging include: Performing mammograms additionally with abnormal result in asymptomatic patients (Yes: 100%, No: 0%) and monitoring of treatments with echocardiograms (every 6 mo, if feasible) in patients who require treatment based in anthracyclines or anti-HER2 agents as a regular assessment of cardiac function (Yes: 78%, No: 22%). In the case of histopathologic diagnosis, examples of medium priority are biopsies for Breast Imaging Reporting and Database System score 4 or 5 Lesions (Yes: 100%, No: 0%) and image-guided (or clinically) biopsy to determine a metastatic relapse (Yes: 100%, No: 0%).

Screening and follow-up are classically considered as low priority. It is safe to deliver these scenarios during the pandemic[6,7] (Yes: 100%, No: 0%) (Table 4).

Priorities for early BC

As high priority in early BC (EBC), all subtypes should complete their regimens that have already started[8]. It is reasonable to consider shorter regimens or dose-modifications (Yes: 100%, No: 0%). Patients with clinical stage (CS) I-II (including N1) and those with intermediate/low grade, "low risk" genetic profile or classified as luminal A subtype do not benefit from neo/adjuvant chemotherapy. These patients can receive endocrine therapy alone (Yes: 100%, No: 0%)[9,10]. Recommendations about subtype are shown in Table 5[8-20].

Examples of medium priority involve situations in HER2 (+) EBC and luminal patients. In BC HER2 (+), anti-HER2 therapy can be restarted after remitting SARS-CoV-2 infection, following a discussion and approval by a multidisciplinary team (Yes:

Table 1 General recommendations for the management of breast cancer

High priority	Comments
(1) Choose regimens and therapeutic sequences consistent with the current situation to reduce the risk of COVID-19 in patients and health workers (Yes: 100%, No: 0%); (2) Differentiated triage for COVID-19 in all cancer centers before entrance (Yes: 100%, No: 0%); (3) Leave a medical register (informed consent) about the discussion of risk/benefits of treatments as well as therapeutic decisions and alternatives available (Yes: 78%, No: 22%); and (4) Individualize the need for blood transfusions when strictly necessary (Yes: 89%, No: 11%)	(1) These recommendations will be adapted according to the reality of each oncological center; (2) Treatment decisions are based on protocols (international/local) about the management of COVID-19; and (3) Multidisciplinary web meetings are recommended to decide the best choices of treatments and outcomes

COVID-19: Coronavirus disease 2019.

Table 2 Priorities of outpatient visits for breast cancer during the coronavirus disease 2019 pandemic

High priority	Medium priority	Comments
(1) Unstable postsurgical patients (hematoma, infection, bleeding) (Yes: 100%, No: 0%); (2) Oncological emergencies (febrile neutropenia, uncontrolled pain, symptomatic brain metastases) (Yes: 100%, No: 0%); (3) BC diagnosis during pregnancy (Yes: 100%, No: 0%); and (4) *De novo* locally advanced BC with aggressive phenotypes (HER2, TNBC) (Yes: 78%, No: 22%)	(1) *De novo* invasive BC (during the multidisciplinary evaluation, priority is guided by tumor biology and clinical stage) (Yes: 89%, No: 11%); (2) Intercurrences on-treatment patients (new signs/symptoms, abnormal findings during physical examination, adverse events (Yes: 100%, No: 0%); (3) Patients on active intravenous chemotherapy (Yes: 78%, No: 22%); and (4) Stable routine postsurgical patients (Yes: 100%, No: 0%)	In patients requiring urgent clinical evaluation, consider converting to telemedicine for follow-up, according to medical evolution

BC: Breast cancer; HER2: Human epidermal growth factor receptor 2; TNBC: Triple-negative BC.

Table 3 Priorities for telemedicine

Medium priority	Low priority	Comments
(1) During the pandemic, most patients could be evaluated using telemedicine (if feasible) (Yes: 100%, No: 0%); and (2) BC patients who can be evaluated with telemedicine: (a) Patients completing neoadjuvant chemotherapy and waiting for surgery (Yes: 89%, No: 11%); (b) Patients eligible for radiotherapy (Yes: 89%, Abst: 11%); and (c) Patients receiving oral chemotherapy or endocrine therapy + targeted therapy (Yes: 78%, No: 22%)	Patients can be evaluated with telemedicine (including after pandemic is over): (a) Routine evaluations in patients who are in periodic controls (observation) or endocrine therapy (Yes: 78%, No: 22%); (b) Survivorship follow-up (Yes: 100%, No: 0%); (c) Psychological visits (Yes: 100%, No: 0%); and (d) New diagnosis of non-invasive BC (Yes: 100%, No: 0%)	(1) During follow-up of patients with high-risk of recurrence, an in-person visit can be assessed according to evolution (if necessary); and (2) In some oncological centers, it is possible to evaluate in-person oral treatments of continuing patients (including whom with adjuvant therapy)

BC: Breast cancer.

78%, No: 22%). In luminal EBC in postmenopausal CS I patients with low/intermediate grade tumors or lobular breast histology variants, endocrine therapy may be started when surgery is deferred (up to 6-12 mo) as indicated in clinical practice guidelines (Yes: 100%, No: 0%). For patients with low-risk genomic score/signature, endocrine therapy should be started alone (Yes: 89%, No: 11%).

Priorities for neoadjuvant medical treatment of BC

All the recommendations in these settings are considered high priority. BC patients initiating therapy or ongoing neo/adjuvant chemotherapy and who present suspected symptoms of infection or contact history with an infected person are recommended to get tested (polymerase chain reaction) before starting or continuing it. In the case of a positive result, defer treatment until confirmation of negative result with a new test (polymerase chain reaction), which could be performed between 2-3 wk later, and with previous evaluation from the Infectious Disease Department (Yes: 67%, No: 33%).

Neoadjuvant treatment according to subtypes: A multidisciplinary team (using web platforms) should evaluate patients with invasive BC for the decision to initiate neoadjuvant therapy during the pandemic (Yes: 100%, No: 0%). Neoadjuvant treatment in BC with "high risk" [triple-negative, HER2 (+), luminal B with "high risk"] is recommended (Yes: 100%, No: 0%)[21,22]. In patients with HR (+) BC, neoadjuvant endocrine therapy allows deferring definitive surgery[23-25] (Yes: 100%, No: 0%). Recommendations about subtypes are included in Table 6[26].

Table 4 Priorities for diagnostic and imaging of breast cancer

High priority	Medium priority	Low priority	Comments
(1) Clinical diagnosis: (a) Diagnosis of a mass or lump (auto examination) or other signs with high suspicion of malignancy (Yes: 100%, No: 0%); and (b) Clinical evidence of relapsed locoregional disease (Yes: 100%, No: 0%); (2) Imaging: (a) Urgent situations that require imaging (oncological emergencies, serious postsurgical complications, *etc*.) (Yes: 100%, No: 0%); (b) Perform additional images upon abnormal mammogram results or suspected metastasis (depending on clinical stage and tumor biology) (Yes: 89%, Abst: 11%); and (c) Images for relapsed BC (Yes: 100%, No: 0%); and (3) Pathological diagnosis: Pathologic evaluation (cytopathology or histopathology) for abnormal mammograms or symptoms in the breast or symptomatic metastatic relapse (Yes: 100%, No: 0%)	(1) Imaging: (a) Perform additional images upon abnormal mammogram results or suspected metastasis (depending on the clinical stage and tumor biology) (Yes: 100%, No: 0%); and (b) Echocardiograms (every 6 mo, if feasible) in patients who require treatment based in anthracyclines or anti-HER2 agents (Yes: 78%, No: 22%); (2) Pathological: (a) Biopsy for BIRADS 4 or 5 lesions (Yes: 100%, No: 0%); and (b) Image-guided (or clinically) biopsy to determine a metastatic relapse (note: metastatic relapses should not be 100% biopsies) (Yes: 100%, No: 0%)	(1) Screening: All screening exams (mammograms or images) for symptomatic patients (*e.g.*, ultrasound or MRI) may be performed after pandemic (Yes: 100%, No: 0%)—BRCA mutated carriers < 40 yr may be considered for screening if delays or more than 6 mo are expected[6,7]; and (2) Follow-up: (a) EBC patients that require images, reevaluation of disease, echocardiograms, and bone scans, should be deferred if patients are clinically asymptomatic (Yes: 100%, No: 0%); and (b) In MBC patients, follow-up oriented to symptoms is recommended. Images, disease reevaluation, echocardiograms may be deferred in large intervals (Yes: 100%, No: 0%)	All patients with a new mass lump with a high suspicion of malignancy or who have already undergone imaging with a high suspicion for malignancy (*e.g.*, BIRDAS 5 in mammogram) should be immediately referred for histological diagnosis and imaging, as a high priority

BC: Breast cancer; BIRADS: Breast Imaging Reporting and Database System score; EBC: Early BC; MBC: Metastatic BC; MRI: Magnetic resonance imaging.

Table 5 Prioritization for early breast cancer

High priority	Medium priority	Comments
(1) General considerations: (a) All subtypes should complete their regimens that have already started[8]. Consider shorter regimens or dose-modifications (Yes: 100%, No: 0%); and (b) Patients with CS I-II (including N1) and those with intermediate/low grade, "low risk" genetic profile, or classified as luminal A subtype do not benefit from neo/adjuvant chemotherapy. They can receive endocrine therapy alone[9,10] (Yes: 100%, No: 0%); (2) TNBC EBC: (a) Chemotherapy neo/adjuvant is recommended. Sequential treatment with one agent reduces complications[11] (Yes: 100%, No: 0%); and (b) Adjuvant capecitabine is recommended in post-neoadjuvant residual disease (Yes: 100%, No: 0%); (3) HER2 (+) EBC: (a) Neo/adjuvant systemic therapy + targeted therapy anti-HER2 is recommended (Yes: 100%, No: 0%); (b) Complete neo/adjuvant chemotherapy (+/- anti-HER2 therapy) for HER2 (+) EBC (Yes: 100%, No: 0%); (c) Trastuzumab-based adjuvant therapy could be reduced from 12 mo to 6 mo without affecting outcomes[12,13] (Yes: 100%, No: 0%); (d) Consider the use of T-DM1 (+/- pertuzumab) in the neo/adjuvant scenario (reduces risk of neutropenia, hospital admissions, use of corticosteroids)[14-16] (Yes: 67%, No: 22%, Abst: 11%); and (e) Continue with T-DM1 in HER2 (+) patients as an adjuvant therapy for post-neoadjuvant residual disease[17,18] (Yes: 100%, No: 0%); and (4) Luminal EBC: (a) Neo/adjuvant endocrine therapy +/- chemotherapy for HR (+)/HER2 (-) BC is recommended (Yes: 100%, No: 0%); (b) Continue standard adjuvant endocrine therapy in pre and postmenopausal patients (use telemedicine to manage potential toxicities reported by patients) (Yes: 100%, No: 0%); and (c) Neoadjuvant endocrine therapy is an option for HR (+)/HER2 (-) BC patients allowing to defer surgery between 6-12 mo in BC with CS I or II (Yes: 100%, No: 0%)	(1) HER2 (+) EBC: Anti-HER2 therapy can be restarted after remitting SARS-CoV-2 infection, following a discussion, and approval by a multidisciplinary team (Yes: 78%, No: 22%); and (2) Luminal EBC: (a) In postmenopausal CS I patients with low/intermediate grade tumors or lobular BC, endocrine therapy may be started when surgery is deferred (Yes: 100%, No: 0%); and (b) For patients with low-risk genomic score/signature, endocrine therapy should be started alone (Yes: 89%, No: 11%)	(1) In patients with active infection due to COVID-19, stopping treatment is recommended; (2) Antihormonal therapy: Endocrine therapy (tamoxifen, AI, LHRH agonists) is safe (does not affect the immune system) and can be continued during the COVID-19 pandemic. LHRH analogs can be administered every 3 mo (although home administration of LHRH analogs is the preferred recommendation); however, fulvestrant requires intramuscular monthly application[19]; (3) Chemotherapy: schedules can be modified to reduce admissions (every 2 or 3 wks' doses can be used instead of a weekly dose with selected agents). Consider the use of concomitant colony-stimulating factor (G-CSF), preferably pegfilgrastim for single-dose administration)[20]; and (4) Bone modifying agents (denosumab or bisphosphonates) can be deferred or administrated every 3 mo (without hypercalcemia), both for adjuvant therapy or in long-term treatments

AI: Aromatase inhibitor; BC: Breast cancer; COVID-19: Coronavirus disease 2019; CS: Clinical stage; EBC: Early BC; G-CSF: Granulocyte-colony stimulating factor; HER2: Human epidermal growth factor receptor 2; HR: Hormone receptor; LHRH: Luteinizing hormone-releasing hormone; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

Priorities for adjuvant medical treatment of BC

Similar to neoadjuvant therapy for BC, all the recommendations for adjuvant therapy are the same and considered as high priority (Yes: 100%, No: 0%). Recommendations are mentioned in Table 7.

Priorities for medical treatment in metastatic BC

The goals of treatment in metastatic BC (MBC) are palliative: Improvement of quality of life and prolong survival as well as minimizing adverse effects due to therapy (Yes: 100%, No: 0%). The medical criteria demand the best decision where a delay can result in fatal outcomes (*e.g.*, a patient with visceral crisis needs to start promptly

Table 6 Prioritization for neoadjuvant medical treatment of breast cancer

High priority	Comments
(1) General recommendations: Patients with BC initiating therapy or ongoing neo/adjuvant chemotherapy and who present suspected symptoms of infection or contact history with an infected person is recommended to get tested (PCR) before starting or continuing it. In the case of positive results, defer treatment until confirmation of negative result with a new test (PCR) which could be performed between 2-3 wk later, and with previous evaluation from the Infectious Disease Department (Yes: 67%, No: 33%); (2) Neoadjuvant treatment according to subtypes: (a) A multidisciplinary team (using web platforms) should evaluate patients with invasive BC for the decision to initiate neoadjuvant therapy during the pandemic (Yes: 100%, No: 0%); (b) Neoadjuvant treatment in BC with "high risk" (TNBC, HER2 (+), luminal B with "high risk") is recommended (Yes: 100%, No: 0%); and (c) In patients with HR (+) BC, neoadjuvant endocrine therapy allows deferring definitive surgery (Yes: 100%, No: 0%); (3) TNBC: (a) Standard neoadjuvant chemotherapy is recommended during the pandemic, although regimens that further reduce exposure and toxicity can be accepted (shorter duration, lower risk of immunosuppression and with an interval of every 3 wk) (Yes: 100%, No: 0%); (b) Consider the use of G-CSF (preferably pelfilgastrim for single-dose administration) in conjunction with chemotherapy (Yes: 89%, No: 11%); (c) The reduction of chemotherapy dose does not seem acceptable to reduce the risk of myelosuppression (Yes: 89%, No: 11%); (d) In older patients > 70 yr, the risk/benefit of neoadjuvant chemotherapy should be discussed. Initial surgery (as long as it is available) is an option, hoping that in 4-5 wk the situation improves to start chemotherapy (it should not be postponed more that period) (Yes: 89%, Abst: 11%); and (e) In patients < 70 yr who reject neoadjuvant chemotherapy for fear of COVID-19, initial surgery may be an option (if available) (Yes: 89%, Abst: 11%); (4) HER2 (+): (a) Neoadjuvant chemotherapy associated with anti-HER2 monoclonal antibodies is strongly recommended (Yes: 100%, No: 0%); and (b) A neoadjuvant regimen with dual anti-HER2 blocking for six courses without anthracyclines can be considered[26] (Yes: 67%, No: 11%, Abst: 22%); and (5) Luminal: (a) In those patients who do not accept surgery (initial therapy), the use of neoadjuvant endocrine therapy is considered to delay surgery until the pandemic is over, especially in patients > 70 yr, tumors with high expression of HR and low ki67 (Yes: 100%, No: 0%); (b) In luminal tumors, the benefit of adding neo/adjuvant chemotherapy to endocrine therapy can be estimated by clinic pathological factors: Tumors with histological grade 3, elevated ki67, or axillary involvement benefit more from chemotherapy (Yes: 100%, No: 0%); (c) Genomic platforms (oncotype, mammaprint, prosigna, endopredict) and online tools like ePrognosis provide additional value in establishing the need for chemotherapy (Yes: 100%, No: 0%); (d) In high-risk luminal tumors, neoadjuvant chemotherapy may provide benefits (*e.g.*, an increased rate of conservative surgery) but not increased survival. This should be analyzed with the risk of SARS-CoV-2 infection and the availability of surgery (Yes: 100%, No: 0%); and (e) Recommendations of the use of neoadjuvant chemotherapy are the same as those described for TNBC subtype (Yes: 100%, No: 0%)	In patients with HER2 (+) EBC, trastuzumab can be restarted for up to 6 mo after stopping treatment

BC: Breast cancer; EBC: Early BC; HR: Hormone receptor; PCR: Polymerase chain reaction; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; TNBC: Triple-negative BC.

Table 7 Prioritization for adjuvant medical treatment of breast cancer

High priority	Comments
(1) General recommendations: (a) Recommendations in the neoadjuvant setting are similar to adjuvant therapy (Yes: 100%, No: 0%); (b) In patients > 70 yr, the evaluation of performance status for estimating the risk/benefit of chemotherapy is mandatory. Online tools (such ePrognosis) and genomic profiles can be useful (Yes: 100%, No: 0%); and (c) It is valid to defer the start of adjuvant chemotherapy until the pandemic is over, although decisions should be individualized according to the patient risk and tumoral subtypes (Yes: 100%, No: 0%); (2) TNBC: (a) Adjuvant chemotherapy must be administered in the most effective way (Yes: 100%, No: 0%); (b) It is recommended to initiate adjuvant chemotherapy up to 2 mo after surgery (Yes: 100%, No: 0%); (c) In patients with age > 70 yr, discuss risk/benefits of using adjuvant chemotherapy; consider a regimen less immunosuppressive to avoid hospital admissions (Yes: 100%, No: 0%); (d) Consider the use of concomitant G-CSF to reduce the risk of infections (Yes: 89%, No: 11%); and (e) The use of adjuvant capecitabine, during 6-8 mo, in patients with no PCR after neoadjuvant chemotherapy is recommended (Yes: 100%, No: 0%); (3) HER2 (+): (a) Adjuvant chemotherapy associated with anti-HER2 therapy is recommended (Yes: 100%, No: 0%); (b) Subcutaneous trastuzumab is an alternative to reduce time to hospital admission. (Yes: 100%, No: 0%); (c) In selected patients (low risk, CS I-II, with PCR after neoadjuvant chemotherapy and surgery), it is valid to consider a shorter time of treatment with adjuvant trastuzumab (6 mo) to reduce hospital admission (Yes: 100%, No: 0%); (d) In the case of completing neoadjuvant chemotherapy associated with anti-HER2 therapy, it is reasonable to continue anti-HER2 therapy until surgery (Yes: 89%, No: 11%); and (e) The use of T-DM1 in those patients who cannot reach pCR after neoadjuvant chemotherapy is recommended. Moreover, the use of T-DM1 can be delayed after surgery (Yes: 100%, No: 0%); and (4) Luminal: (a) In "high risk" luminal patients, it is recommended to assess a clinical risk and/or use of genomic platforms (including tumors with lymph node involvement) to limit the use of adjuvant chemotherapy, according to institutional clinical practice guidelines (Yes: 100%, No: 0%); (b) The assessment of clinical risk with online tools (such as Predict) to estimate the risk of recurrence and the benefit of adjuvant chemotherapy is recommended (Yes: 100%, No: 0%); and (c) If adjuvant chemotherapy is necessary (risk/benefit previous evaluation), its initiation can be delayed up to a maximum of 3 mo after surgery (without reducing efficacy) (Yes: 89%, No: 11%)	(1) Delaying adjuvant chemotherapy in TNBC is associated with an increased risk of relapse and death; and (2) To limit the use of adjuvant chemotherapy, luminal BC should be assessed by clinical and/or genomic risks

BC: Breast cancer; CS: Clinical stage; G-CSF: Granulocyte-colony stimulating factor; PCR: Polymerase chain reaction; TNBC: Triple-negative BC.

chemotherapy). The election of chemotherapy, endocrine therapy, targeted therapy, or immunotherapy should be delivered in a selected population [*e.g.*, dual blockade anti-HER2 therapy + chemotherapy in HER2 (+) MBC, if accessible]. Most recommendations are classified as a medium priority in the MBC scenario. Recommendations for medical treatment in MBC are mentioned in Table 8[27-30].

DISCUSSION

The importance to divide recommendations into priorities is a complex challenge in

Table 8 Priorities for medical treatment of metastatic breast cancer

High priority	Medium priority	Low priority	Comments
HER2 (+) MBC: Dual anti-HER2 therapy (pertuzumab/trastuzumab) + chemotherapy is recommended in first-line HER2 (+) MBC (Yes: 100%, No: 0%)	(1) General considerations: (a) Goals for treatment in MBC: improvement of QoL, prolong survival (Yes: 100%, No: 0%); and (b) In absence of clinical infection for SARS-CoV-2, there is no specific recommendation to test a patient who initiates therapy (Yes: 89%, No: 11%); (2) Chemotherapy: (a) Consider the use of less toxic regimens (capecitabine, CM – cyclophosphamide/methotrexate) (Yes: 100%, No: 0%); and (b) If an anthracycline, a taxane, or eribulin is used, consider the concomitant use of G-CSF (Yes: 89%, No: 11%); (3) iCDK 4/6: (a) Offer clinical benefit in the first or second line in luminal MBC. They should be considered according to resources available and institutional practices[27] (Yes: 100%, No: 0%); (b) iCDK 4/6 can be deferred in selected patients if there is a high chance of controlling the disease with endocrine therapy alone (*e.g.*, *de novo* luminal MBC with a low burden of disease without visceral metastases) (Yes: 100%, No: 0%); and (c) Palbociclib dose reduction does not reduce efficacy (Yes: 100%, No: 0%)[28,29]; (4) Visceral crisis: (a) In the case of a suspected visceral crisis, clinical practice guidelines recommend the use of chemotherapy immediately (Yes: 100%, No: 0%); and (b) In an established visceral crisis, chemotherapy is the treatment of choice, but this concept is actually in revision due to the use of iCDK 4/6 in clinical practice (Yes: 100%, No: 0%); (5) Luminal MBC: (a) There should be an analysis of the benefits of using iCDK 4/6 *vs* the risk of adverse events. Dose reduction can reduce treatment related toxicities (Yes: 100%, No: 0%); (b) In the case of premenopausal women, it is recommended to induce ovarian suppression with LHRH analogs monthly or quarterly in selected cases, to reduce hospital admissions (Yes: 100%, No: 0%); (c) First line of treatment: currently, there is insufficient evidence to discontinue treatment with iCDK 4/6 in patients with luminal MBC (Yes: 100%, No: 0%); (d) The second line of treatment: after progression to iCDK 4/6, moving to another endocrine therapy is recommended (*e.g.*, AI or fulvestrant) (Yes: 100%, No: 0%); and (e) Other targeted therapies in luminal MBC: in second-line, consider avoiding or deferring the addition of mTOR inhibitors (everolimus induces immunosuppression and mucositis) or PIK3CA inhibitors (alpelisib induces hyperglycemia and risk of diabetes) to endocrine therapy, especially in elderly and/or comorbid patients (Yes: 100%, No: 0%); (6) HER2 (+) MBC: (a) In patients with more than 2 yr of disease control and minimal tumor burden with trastuzumab-based regimens, discontinuation of maintenance therapy may be considered[30] (Yes: 100%, No: 0%); (b) HR (+)/HER2 (+) MBC: (b1) Patients who initiate first-line treatment: Two options according to age—(i) patients < 70 yr: Consider less toxic regimens (paclitaxel every 3 wk + dual anti-HER2 blockade as a treatment option). If using docetaxel, consider the use of G-CSF from first cycle (Yes: 89%, No: 11%); and (ii) patients > 70 yr: Consider less toxic regimens (capecitabine) + dual anti-HER2 blockade. The option of using endocrine therapy associated to anti-HER2 therapy is valid if there is a low tumoral burden (Yes: 89%, No: 11%); and (b2) patients with controlled disease: Discontinuation of chemotherapy and use of oral therapy with AI +/- LHRH analogs (in premenopausal patients) may be considered as maintenance of anti-HER2 blockade (Yes: 100%, No: 0%); and (c) HR (-)/HER2 (+) MBC: (c1) In first-line setting (where an overall survival > 5 yr is expected) with a patient < 70 yr and symptomatic: Initiate taxanes every 3 wk and/or vinorelbine or capecitabine associated with anti-HER2 blockade (pertuzumab +/- trastuzumab). If using myelosuppressive regimens, consider G-CSF to reduce the risk of neutropenia (Yes: 89%, No: 11%); and (c2) Cases where the disease is in response with one line of chemotherapy + anti-HER2 therapy, in patients classified as "high groups for COVID-19" (*e.g.*, > 65-70 yr and/or comorbidity (diabetes, hypertension, *etc.*): Consider maintaining anti-HER2 therapy as maintenance (Yes: 89%, No: 11%); (7) mTNBC: (a) Recommendations with use of chemotherapy: (a1) Use regimens less frequently and with less toxicity (*e.g.*, use paclitaxel every 2 wk - Q2W- instead of docetaxel Q3W (Yes: 78%, No: 22%); (a2) Consider G-CSF to minimize the risk of neutropenia and reduce the use of corticosteroids (Yes: 89%, No: 11%); (a3) Prefer oral chemotherapy regimens (such as capecitabine) as an alternative to the EV route (if possible) (Yes: 100%, No: 0%); (a4) Consider less immunosuppressive regimens, with a preference for monotherapy regimens rather than combinations (Yes: 100%, No: 0%); and (b) For treatment with targeted therapies, it is recommended: (b1) Immunotherapy: Consider atezolizumab + chemotherapy in patients with PD-L1 (+) mTNBC (Yes: 100%, No: 0%); and (b2) PARP inhibitors (olaparib, talazoparib): Evaluate risk/benefit (myelotoxicity) and drug interactions (Yes: 100%, No: 0%); and (8) Second-line and subsequent lines: (a) Offer second-line, third-line, or subsequent lines of treatment when therapy can deliver clinical benefit and impact on outcomes (Yes: 100%, No: 0%); (b) In HER2 (+) MBC, after progression with trastuzumab, evaluate treatment with T-DM1 and/or other available therapies, always maintaining anti-HER2 blockade (Yes: 100%, No: 0%); (c) Some doses may be deferred to minimize risk of infection during the COVID-19 pandemic (Yes: 100%, No: 0%); and (d) In MBC, after a multidisciplinary discussion and according to the patient's preferences, terms such as "therapeutic rest", palliative therapy or maintenance regimens with dose reduction can be offered, depending on the case (Yes: 89%, No: 11%)	Bone agent therapy: Denosumab and zoledronic acid are no urgently needed (except in cases of hypercalcemia). It can be administered every 3 mo. Bone agents for patients with bone metastases should be deferred (Yes: 100%, No: 0%)	The rapidly evolving nature of the COVID-19 pandemic may change some recommendations. These will evolve over time with continuous updates

AI: Aromatase inhibitor; COVID-19: Coronavirus disease 2019; G-CSF: Granulocyte-colony stimulating factor; HR: Hormone receptor; LHRH: Luteinizing hormone-releasing hormone; MBC: Metastatic BC.

clinical decision-making. The purpose of this guideline is to prioritize patient scenarios by the urgency of treatment and make recommendations based on these priorities without compromising outcomes. This manuscript must be interpreted and adapted according to each oncologist's necessity, prioritizing physician judgment, and is not intended to replace institutional health policies or local guidelines. The goal of this manuscript is to orient and guide health workers related to medical oncology in the development of action plans to maintain the best outcomes for BC patients in the COVID-19 era. Recommendations about "high risk" subtypes (HER2, triple-negative) were similar in guidelines. Conversely, there was certain discordance in luminal subtype, with more flexible options of oncological medical treatment in each scenario (neoadjuvant, adjuvant, early, metastatic). For example, consider the assessment of clinical criteria or genomic platforms for the final decision of therapy in early BC. Consensus was made in order to avoid discordances. The goal of this manuscript is adapting guidelines to local context through relevant decision-makers in order to avoid duplication of efforts and optimize use of medical resources according to the national system[31].

This manuscript was adapted to a Latin America health system. For example, SPOM used some recommendations in order to generate best decisions of oncological medical treatment in Peru.

CONCLUSION

The COVID-19 pandemic represents a unique challenge for patients, clinicians, and the healthcare system. During the COVID-19 pandemic, minimization of exposure risk and preservation of resources are mandatory because there is a need of recommendations according to national realities and possibilities of health services access, without affecting the health of cancer patients. International clinical practice guidelines have formulated guidelines for the management of BC in the COVID-19 era involving all scenarios, most recommendations are similar, mainly in high-risk subtypes (HER2, triple-negative). Certain societies haven adapted these recommendations, including a panel vote with percentages, to deal with different situations involving the best decision in the management of BC patients. This manuscript has used and summarized three clinical practice guidelines, showing a broad vision (American, European) of BC management using different approaches and generating a consensus for adapting to our health system reality.

ARTICLE HIGHLIGHTS

Research background

The evaluation of the reality of access to health services in our country (Peru) is mandatory in coronavirus disease 2019 (COVID-19) era as is the prioritization of different approaches to determine which patients with cancer [in this case with breast cancer (BC)] need immediate treatment or those in which treatment can be deferred until pandemic is over. BC is one of the most frequent tumors in Peru, similar to other countries of Latin America and globally.

Research motivation

In Peru there is no information about a consensus or local guidelines of BC and COVID-19 management.

Research objectives

The main objective is to adapt international clinical practice guidelines to local context through best decision-makers, avoid duplication of efforts, and optimize medical resources, since Peru has many limitations to access a optimal health care attention.

Research methods

Peruvian Society of Medical Oncology invited an expert panel (including nine medical oncologists, opinion leaders in Peru) who reviewed and were asked to cast their vote. Decisions were discussed with a consensus though teleconference. One hundred twelve recommendations were reviewed, and priority categories were defined and adapted in three levels based on the severity and urgency of an individual patient's

conditions or treatments. Finally, some comments were allowed in each topic discussed.

Research results

Recommendations about human epidermal growth factor receptor 2 and triple-negative subtypes were quite similar, but in the luminal subtype there are more options for clinical decisions since treatment of different scenarios (mainly metastatic BC) has evolved in the last few years.

Research conclusions

Majority of recommendations were reviewed and adapted from international clinical practice guidelines to local context.

Research perspectives

The authors believe that this manuscript will be useful for other Latin America countries to adapt health policies in the COVID-19 era.

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EDITORIAL

Metastatic hormone-sensitive prostate cancer: How should it be treated?

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Abstract

The number of treatment options for metastatic hormone-sensitive prostate cancer has increased substantially in recent years. The classic treatment approach for these patients—androgen-deprivation therapy alone—is now considered suboptimal. Several randomized phase III clinical trials have demonstrated significant clinical benefits—including significantly better overall survival and quality of life—for treatments that combine androgen-deprivation therapy with docetaxel, abiraterone acetate, enzalutamide, apalutamide, and/or radiotherapy to the primary tumour. As a result, these approaches are now included in treatment guidelines and considered standard of care. However, the different treatment strategies have not been directly compared, and thus treatment selection remains at the discretion of the individual physician or, ideally, a multidisciplinary team. Given the range of available treatment approaches with varying toxicity profiles, treatment selection should be individualized based on the patient's clinical characteristics and preferences, which implies active patient participation in the decision-making process. In the present document, we discuss the changing landscape of the management of patients with metastatic hormone-sensitive prostate cancer in the context of several recently-published landmark randomized trials. In addition, we discuss several unresolved issues, including the optimal sequencing of systemic treatments and the incorporation of local treatment of the primary tumour and metastases.

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Core Tip: Due to advances in the treatment of metastatic hormone-sensitive prostate cancer in recent years, multiple options are now available. The emergence of androgen receptor inhibitors provides patients with an alternative to chemotherapy. Given the increasingly important role of these novel treatments, a comprehensive review of the available data is needed. In addition, there are several unresolved questions and controversies surrounding these treatments, which can only be resolved by in-depth analysis and consensus among the specialists who treat these patients.

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INTRODUCTION

In 1941, the researchers Huggins and Hodges demonstrated, for the first time, that it was possible to reduce tumour volumes in prostate cancer (PCa) and improve disease-related symptoms through orchiectomy or estrogen therapy. This important finding was later recognized with the Nobel Prize in Physiology. Since then, androgen-deprivation therapy (ADT) has been the standard therapy for patients with newly-diagnosed metastatic PCa. In most cases, ADT is achieved with luteinizing hormone-releasing hormone (LHRH) analogues (leuprorelin, goserelin, and/or triptorelin)[1]. While the use of LHRH antagonists is less common, it may increase in the near future due to the recent development of oral LHRH antagonists (*e.g.*, relugolix)[2].

The estimated initial response rate of the primary tumour and metastases to ADT ranges from 60% to 80%[3,4]. However, the prognosis of patients with metastatic disease is poor. For this reason, various clinical trials have been conducted in recent years to evaluate the benefit of treatment intensification in these patients[5,6].

Randomized trials performed in the year 2013 were mainly focused on determining the relative value of intermittent *vs* continuous ADT in selected patients[7]. However, just a few years later, the positive results of two phase III trials, the CHAARTED trial and the multi-arm STAMPEDE trial (arm C), both of which confirmed the superiority of docetaxel plus ADT, led to a paradigm shift. Consequently, this combined treatment approach became the new standard of care in selected patients[8,9]. In the CHAARTED trial, patients were stratified according to disease volume (high *vs* low). High-volume metastatic disease was defined as the presence of visceral metastases and/or ≥ 4 bone lesions (with at least one located outside of the vertebral column or pelvis). Patients who did not meet at least one of these two criteria were considered to have low-volume disease[8]. This definition is important given the benefits observed in overall survival (OS) in patients with high-volume disease (CHAARTED criteria) treated with docetaxel. In fact, the main European guidelines[10] now recommend docetaxel in these patients. However, conclusive data regarding the magnitude of effect of this treatment in patients with low-volume disease are not available[11,12]. Despite the limitations of the CHAARTED definition, it has been subsequently used in the design of clinical studies in the same clinical setting[13-15].

Since then, we have witnessed the successive publication of five randomized clinical trials in which classic ADT (LHRH analogues) combined with androgen receptor signaling inhibitors (ARSi) were compared to ADT alone or, in some trials, to first-generation anti-androgens[13-17]. The positive results of those trials supporting treatment intensification (*i.e.* combination therapy) raises new questions regarding the superiority of ARSi *vs* docetaxel and how to select the most appropriate ARSi for each patient.

Abiraterone acetate was the first ARSi to demonstrate efficacy in metastatic PCa.

The findings of the LATITUDE[16] and STAMPEDE trials (arm G)[17] demonstrated that abiraterone acetate combined with ADT significantly reduced mortality risk while prolonging radiographic progression-free survival (rPFS). The long-term outcomes (median follow-up, 51.8 mo) of the LATITUDE trial were recently reported, confirming the initial findings of the trial and showing that 66% of patients treated with combination therapy survived at 5 years[18]. Subsequent quality of life (QoL) and cost-effectiveness studies further supported these findings; as a result, abiraterone acetate became the first ARSi approved for the treatment of metastatic hormone-sensitive PCa (mHSPC). Although there were differences between the two trials in terms of the type of metastatic patients (high risk *de novo* PCa in LATITUDE and any metastatic or non-metastatic patient in the STAMPEDE trial), the results of both trails showed that combination therapy provided a clinical benefit in all patient profiles[19]. However, the Food and Drug Administration (FDA) and the European Medicines Agency only approved this treatment for *de novo* high-risk patients, defined in the LATITUDE trial[18] as patients presenting at least two of the following three characteristics: ≥ 3 bone lesions; measurable visceral disease; and Gleason score ≥ 8. In these patients, median survival (53.3 mo) was virtually identical to that obtained with docetaxel (51.2 mo) in the CHARTEED trial[8] in patients with high-volume disease, even though the patient profiles in those two trials differed substantially, making it difficult to directly compare the results from these two trials.

The phase III ENZAMET[13] and ARCHES[14] trials, both published in 2019, confirmed the value of enzalutamide in patients with mHSPC, thus supporting the use of enzalutamide plus ADT in these patients. Although the clinical profile of the patients in the two studies was similar, there were clear differences in study design (Table 1). For example, in the ENZAMET study[13] patients were allocated to receive either enzalutamide plus ADT or first-generation antiandrogens plus ADT; by contrast, the ARCHES[14] trial compared enzalutamide plus ADT to ADT plus placebo. Similarly, there were also differences in the primary endpoints: OS in the ENZAMET trial and rPFS in the ARCHES trial.

At a median follow-up of < 36 mo, the results of the ENZAMET trial confirmed a benefit for the study drug (improved rPFS) in all subgroups, with a 3-year OS of 80% in the enzalutamide plus ADT arm *vs* 72% in the comparison arm (ADT plus first-generation antiandrogens [bicalutamide, flutamide, or nilutamide]). Based on these results, the FDA approved enzalutamide for the treatment of mHSPC in December 2019. However, the European Medicines Agency considered that the survival data are immature and has not, therefore, added this indication to the drug label.

The TITAN[15] study, a randomized clinical trial involving 1052 patients, confirmed the clinical utility of apalutamide, which now forms part of the therapeutic arsenal for the treatment of mHSPC. At a median follow-up of 22.7 mo, both OS and rPFS were comparable to outcomes obtained with abiraterone acetate and enzalutamide in patients with a similar clinical profile (Table 2). At 2 years of follow-up, there was a significant reduction in both mortality risk (33%) and radiographic progression (52%). Based on these data, the FDA approved treatment in June 2019, with the following indication on the drug label "in adult men for the treatment of mHSPC in combination with androgen deprivation therapy". However, the risk groups were not specified on the drug label, in contrast to abiraterone acetate, due to the broader inclusion criteria of the TITAN trial.

With regard to ARSi in patients with low-volume disease, patients in the ARCHES and ENZAMET trials[13,14] were stratified by disease burden (high *vs* low-volume disease, CHAARTED criteria). The findings of those trials provided further support for the potential utility of ARSi in patients with low-volume disease beyond the usual treatment indication in high-volume, high risk patients.

The optimal treatment for mHSPC remains controversial, mainly because abiraterone acetate and docetaxel yield comparable results. However, the expected regulatory approval of apalutamide and enzalutamide (pending regulatory processes in multiple regions) is likely to end this debate. Most patients diagnosed with mHSPC are asymptomatic at diagnosis, which is why it is important to maintain QoL in these patients. A recent comparison of QoL in patients in arms C (docetaxel + ADT) and G (abiraterone acetate + ADT) in the STAMPEDE trial showed better results for abiraterone acetate, especially in the first year treatment[20]. Patients must be informed of these relevant results, which should be taken into account in the treatment selection process.

There is also some controversy surrounding the treatment indication in patients with significant comorbidities given that their systematic exclusion from most phase III trials. As a result, treatment selection in these cases largely depends on studies with lower levels of evidence and on the treating specialist's experience with these drugs in

Table 1 Characteristics of the randomized phase 3 trials of second-generation antihormonal treatments in metastatic hormone-sensitive prostate cancer

Trial	Treatment	*n*	Inclusion criteria	Stratification	Main objective	Secondary objectives
LATITUDE (Fizazi K *et al*[16]) 2017	ABIRATERONE 1000 mg + PREDNISONE 5 mg + ADT; PLACEBO + ADT	597; 602	High risk con ≥ 2 factors: (1) Gleason 8-10; (2) ≥ 3 bone METS; (3) Evaluable visceral METS; and (4) ECOG PS ≤ 2	Visceral METS (yes/no); ECOG PS (0, 1/2)	OSrPFS	Time to pain progression; Time to PSA progression; Development of skeletal events; Time to chemotherapy; Time to new treatment; QoL
STAMPEDE (James *et al*[17])[1] 2017	ABIRATERONE 1000 mg + PREDNISONE 5 mg + ADT; ADT	502; 500	Presence of METS	ECOG PS (0/1, 2); AGE (< 70/≥ 70); Use of steroids (yes/no); Research centre; Indication for RT (yes/no); Type of ADT	OS	PFS; Failure-free survival; Cancer-specific survival; Time to skeletal events; Toxicity; QoL
ENZAMET (Davis *et al*[13]) 2019	ENZALUTAMIDE 160 mg + ADT; ANTIANDROGENS (bicalutamide, flutamide or nilutamide) + ADT	563; 562	Low and high volume defined: (1) Visceral METS; and (2) ≥ 4 bone METS, at least 1 outside vertebral column or pelvis. ECOG PS ≤ 2	Volume (high/low); Docetaxel (yes/no); Comorbidities (0-1/2-3); Antiresorptive treatment (yes/no); Research centre	OS	PFS; PSA response; Adverse effects; QoL; Cost-effectiveness
TITAN (Chi *et al*[15]) 2019	Apalutamide 240 mg + ADT; PLACEBO + ADT	525; 527	LOW AND HIGH VOLUME DEFINED:(1) Visceral METS + ≥ 1 bone METS; and (2) ≥ 4 bone METS, at least 1 outside vertebral column or pelvis. ECOG PS 0-1; ≥ 1 bone METS on bone scan	Gleason (≤ 7/≥ 8); Docetaxel (yes/no); Geographic region	OS; rPFS	Time to pain progression; Time to opioids; Time to skeletal events; Time to chemotherapy; Time to PSA progression; Survival to 2nd progression; Time to symptomatic local progression; QoL
ARCHES (Armstrong *et al*[14]) 2019	ENZALUTAMIDE 160 mg + ADT; PLACEBO + ADT	574; 576	ECOG PS 0-1; low and high volume defined:(1) Visceral METS; and (2) ≥ 4 bone METS, at least 1 outside vertebral column or pelvis	Volume (high/low); Docetaxel (no/0-5 cycles/≥ 6)	rPFS; Evaluated at 24 wk	Time to PSA progression; Time to skeletal events; Time to new treatment; Undetectable PSA; Objective response rate; OS; Time to worsening of urinary symptoms; Time to pain progression; Time to castration-resistance; Time to QoL worsening; PROS

[1]Only patients with metastases. ADT: Androgen deprivation therapy; METS: Metastases; OS: Overall survival; PROs: Patient-reported outcomes; PS: Performance status; PSA: Prostate-specific antigen; QoL: Quality of life; rPFS: Radiographic progression-free survival; RT: Radiotherapy.

other clinical settings for which they are approved. In any case, an individualized assessment of each patient should be performed to determine the most appropriate treatment, which should also take into account differences in the side-effect profiles of second-generation antihormonal therapies. Similarly, cost-effectiveness and the optimal sequencing of these drugs with docetaxel are also key factors to consider[21,22]. Unfortunately, the optimal sequence is not known due to the lack of data from randomized clinical trials. The mechanism of action of docetaxel differs from that of second-generation antiandrogens. Therefore, administration of docetaxel in patients with progressive disease after treatment with these second-generation antiandrogens would conceivably eliminate treatment-resistant cell clones (regardless of the specific androgen receptor pathway), thereby permitting the use of another ARSi in patients who develop progression.

Treatment of the primary tumour with surgery or radiotherapy plays an important role in treatment outcomes. The role of radiotherapy to the primary was evaluated in both the HORRAD[23] and STAMPEDE trials (arm H)[24], adding another dimension to the discussion about the indication for radiotherapy in metastatic disease. The available evidence for surgical treatment of the primary tumour in this clinical setting is contradictory, and the evidence to support its use is weak[25,26]. However, the role of surgery is currently being evaluated in large trials, which will help to determine the value of this approach in this clinical setting. By contrast, the results of the STAMPEDE trial (arm H) confirmed a statistically significant benefit for radiotherapy to the primary in terms of improved 3-yr OS (increase of 8%) in patients with low-volume disease (CHAARTED criteria). Even though the study design did not stratify patients into low- and high-volume disease, an in-depth analysis of the trial results confirmed the validity of stratification according to metastatic burden, leading the

Table 2 Survival and toxicity results of the phase 3 randomized clinical trials of second-generation antihormonal treatments in metastatic hormone-sensitive prostate cancer

Trial	Follow-up in mo	Main results	Quality of life	Toxicity
LATITUDE	51.8	Reduced mortality risk by 34% with abiraterone ($P < 0.001$); OS at 3 yr (66% *vs* 49%); median OS (53.3 mo *vs* 36.5 mo). Reduction in risk of radiographic progression by 53% with abiraterone ($P < 0.001$) (33 mo *vs* 14.8 mo)	Abiraterone improved all QoL-related parameters	Toxicity grade 3-4: (1) Abiraterone: 63%; (2) Placebo: 48%. Treatment-related deaths: (1) Abiraterone: 5%; and (2) Placebo: 4%
STAMPEDE	40	Reduction in mortality risk of 39% with abiraterone ($P < 0.001$). Reduction in risk of progression of 69% with abiraterone ($P < 0.001$). Treatment benefit in all patients according to risk group and disease volume	-	Toxicity grade ≥ 3: (1) Abiraterone: 47%; and (2) ADT: 33%
ENZAMET	33	Reduction in mortality risk by 67% with enzalutamide ($P = 0.002$). 3-yr OS (82% *vs* 72%)	No SD	Toxicity grade ≥ 3: (1) Enzalutamide: 58%; and (2) AA: 43%
TITAN	22.7	Reduction in risk of radiographic progression or death by 52% with apalutamide ($P < 0.001$). 2-yr rPFS (68.2% *vs* 47.5%). Reduction in mortality risk of 33% with apalutamide ($P = 0.005$). 2-yr OS (82.4% *vs* 73.5%). Treatment benefit in all patients according to disease volume, significant in rPFS and OS in high-volume disease	No SD	Toxicity grade 3-4: (1) Apalutamide: 42.2%; and (2) Placebo: 40.8%. Treatment-related deaths: (1) Apalutamide: 1.9%; and (2) Placebo: 3%
ARCHES	14.4	Reduction in risk of radiographic progression or death of 61% with enzalutamide ($P < 0.001$) (NR *vs* 19 mo). OS ($P = 0.336$). Benefit in rPFS in both high and low volume disease	No SD	No SD. Treatment-related deaths: (1) Enzalutamide: 2.4%; and (2) Placebo: 1.7%

AA: First-generation antiandrogens; ADT: Androgen deprivation therapy; NR: Not reached; OS: Overall survival; QoL: Quality of life; rPFS: Radiographic progression-free survival; SD: Statistical differences.

authors to conclude that radiotherapy to the primary should be considered the new standard of care in low-volume mHSPC given that it does not worsen local symptomatic events[27]. However, there is some uncertainty regarding the magnitude of benefit of radiotherapy in patients who receive systemic treatment with docetaxel, abiraterone acetate, enzalutamide, or apalutamide given that only 18% of patients in the H arm of the STAMPEDE trial received chemotherapy, and none received second-generation antihormonal therapy. In this regard, the results of the PEACE-1 trial (NCT01957436) are expected to resolve this question when they are published.

Another important unresolved question—despite a growing body of favourable data—is the role of stereotactic body radiotherapy to the bone and/or lymph node metastases in oligometastatic patients. Conceivably, stereotactic body radiotherapy should complement the positive effects of radiotherapy to the primary combined with systemic therapy, although the magnitude of the benefit of administering all three treatments remains unknown.

CONCLUSION

Androgen receptor inhibitors, whose clinical utility was first demonstrated in advanced PCa, now are part of the therapeutic arsenal for mHSPC. Given the demonstrated superiority ARSi plus ADT *vs* ADT alone, the indication for ADT monotherapy is now limited to patients with significant comorbidity and a short life expectancy, or to patients who refuse combination therapy, despite the clear benefits.

Chemotherapy has shown a clear benefit as initial treatment in patients with high-volume disease. However, selection of the "optimal" treatment must take into account the toxicity profile of the treatment as well as its impact on QoL, especially given the wide array of alternative treatments—including abiraterone acetate, enzalutamide, and apalutamide—all of which have shown excellent clinical results with substantially less toxicity than chemotherapy and thus less of a negative impact on QoL.

Currently, radiotherapy to the primary tumour is considered the new standard of care in patients with low-volume disease, at least until results are published to clarify the impact of treatment intensification, including both the treatment of metastatic lesions and the combination of local treatments with novel systemic therapies.

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EDITORIAL

New Year's greeting and overview of *World Journal of Clinical Oncology* in 2021

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Abstract

Although the whole world has been suffering under the Coronavirus Disease 2019 epidemic, the *World Journal of Clinical Oncology* (*WJCO*) was able to productively direct our efforts in the field through 2020. This was only accomplished through the collective efforts and support of our Editorial Board members, peer reviewers, authors, readers and editorial office staff. In 2020, the *WJCO* published 12 issues encompassing 84 papers, a 115.4% increase over 2019 (39 papers). In December of 2020, we strategized a plan to solicit even more high-quality contributions for *WJCO* in 2021. So far, we have registered and accepted 188 manuscript titles, including 132 reviews and 56 original articles. In the New Year, we will work with our internal and external colleagues who care about and support the development of *WJCO* with the express aim of improving the academic rank of *WJCO* in the field of oncology. Ultimately, our goal is to merit and gain inclusion in the Science Citation Index Expanded, with the first impact factor being awarded as soon as possible.

Key Words: *World Journal of Clinical Oncology*; Publication; Editorial board; Oncology

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Core Tip: This editorial introduces the development of *World Journal of Clinical Oncology* (*WJCO*) in 2020. We also discuss some of the limitations currently facing the journal and propose solutions that can positively influence the academic rank of *WJCO*. Finally, we thank all those who have contributed to the development of *WJCO* and look forward to continuing our collective efforts to strengthen the field of clinical

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INTRODUCTION

The *World Journal of Clinical Oncology* (*WJCO*, *World J Clin Oncol*) was launched in 2010 as a high-quality, monthly, online, open-access, single-blind, peer-reviewed journal published by the Baishideng Publishing Group[1]. Since then, the *WJCO* has been abstracted and indexed in PubMed, PubMed Central[2], and Emerging Sources Citation Index (Web of Science). This journal mainly publishes basic or clinical research in the field of oncology, and the types of articles published in *WJCO* include Review, Original Article, Case Report, *etc*.[1].

ACHIEVEMENTS OF *WJCO* IN 2020

As editors of the *WJCO*, it is our great pleasure to take this opportunity to wish all of our authors, readers, Editorial Board members, independent expert referees, and staff of the editorial office a Very Happy New Year. On behalf of the Editorial Team, we would like to express our gratitude to all authors who have contributed their valuable manuscripts, to the independent referees, and to our readers for their continuous support, dedication, and encouragement. The collective team effort by our Editorial Board members and Editorial Office staff allowed the *WJCO* to advance further in 2020.

Published articles in 2020

The *WJCO* published 12 issues in 2020, encompassing 84 articles. These articles included 29 unsolicited manuscripts (34.5%, 29/84) and 55 invited manuscripts (65.5%, 55/84). Of these manuscripts, there were 22 original articles (26.2%, 22/84), 40 review articles (47.6%, 40/84), 5 editorials (6.0%, 5/84), 16 case reports (19.0%, 16/84), and 1 'other' type (1.2%, 1/84) (Figure 1). Overall, the number of publications of *WJCO* in 2020 represented a remarkable increase over the previous year (115.4%, 39 more articles).

Metrics for manuscripts submitted and published in 2020

In 2020, the *WJCO* received a total of 125 manuscript submissions for consideration of publication after peer review. Among them, 107 received a first decision by the Science Editor Development Department with 18 (14.4%, 18/125) being rejected and 53 (45.2%, 53/125) ultimately being published online by the Production Department. Those 53 articles included 31 invited manuscripts (58.5%, 31/53) and 22 unsolicited manuscripts (41.5%, 22/53). Among them were 12 original articles (22.6%, 26/53), 25 reviews (47.2%, 25/53), 4 editorials (7.5%, 4/53), 11 case reports (20.7%, 11/53), and 1 'other' type article (1.9%, 1/53). The authors hailed from 22 countries and regions mostly from the United States (24.5%, 13/53), Spain (15.1%, 8/53), and China, Japan, and Italy (5.7%, 3/53); various other countries or regions accounted for the remaining 23 articles (43.4%, 23/53) (Figure 2).

Peer review of manuscripts

In 2020, the *WJCO* invited a total of 4379 peer reviewers and Editorial Board members to review manuscripts. These invitations garnered 645 (14.7%, 645/4379) acceptances to perform the peer review, 697 (15.9%, 697/4379) declinations to perform the peer review, and 2648 (60.5%, 2648/4379) nonresponses. Among the 645 reviewers who accepted the invitation, 234 (36.3%, 234/645) submitted peer review reports, 398 (61.7%, 425/645) failed to submit peer review reports, and 13 (2.0%, 13/645) have not yet submitted the peer review report.

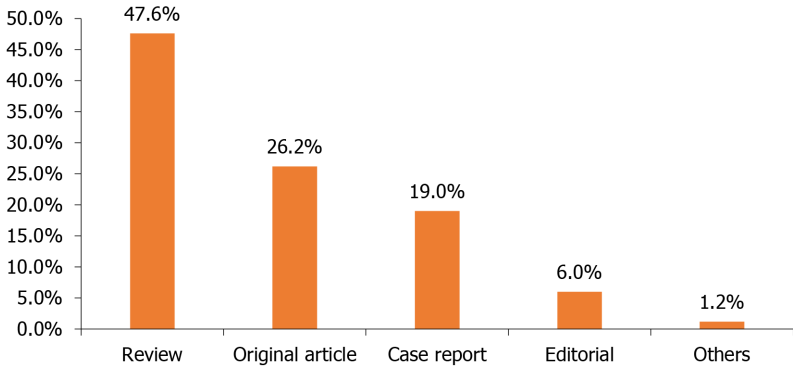


Figure 1 Column type distribution of manuscripts published in *World Journal of Clinical Oncology* in 2020.

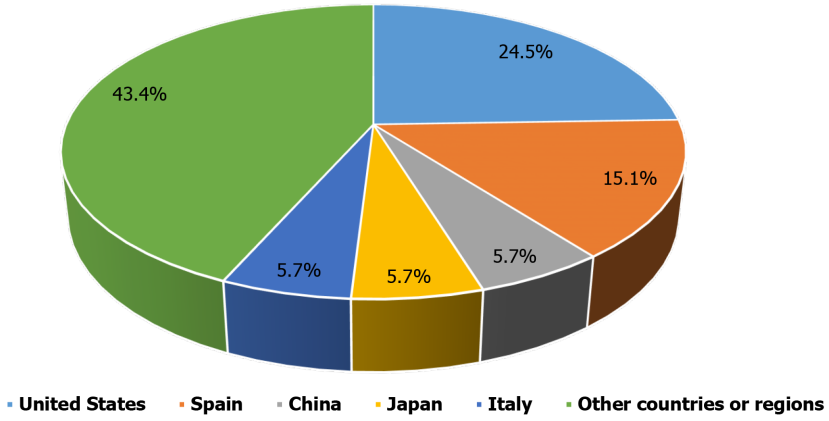


Figure 2 Distribution of authors' countries for the manuscripts published in *World Journal of Clinical Oncology* in 2020.

Citation

As of January 10, 2021, according to data from the Web of Science, the *WJCO* published 64 articles in 2018 (25) and 2019 (39) from authors in 27 countries or regions. These 64 published articles included 55 classified as "article" or "review" and were cited 166 times in 2020.

Editorial Board members

In 2020, the Editorial Board of *WJCO* was composed of 73 members from 23 countries and regions[3], including 12 (16.4%) from China, 12 (16.4%) from the United States, 9 (12.3%) from Italy, 4 (5.5%) from Greece, 4 (5.5%) from India, and 32 (43.8%) from various other countries and regions (Figure 3). Considering the small number of Editorial Board members and the uneven distribution of regions and disciplines, the Editorial Office will invite more experts from the oncology field to join the *WJCO* Editorial Board in 2021.

Soliciting contributions

By the end of 2020, the Editorial Office of *WJCO* had formulated a plan for soliciting contributions from authors in 2021. We have now invited the Editorial Board members and high-level scientists in the field of oncology to solicit contributions for reviews and original articles. So far, 188 invited manuscript titles have been registered by authors and accepted for upcoming submission and review of the full papers; these have included proposals for 132 review articles and 56 original articles. The solicitation of original articles is ongoing.

LIMITATIONS AND SOLUTIONS

Although the *WJCO* achieved phased success in 2020, it has not yet reached our pinnacle goals. At present, the *WJCO* still faces some challenges that need to be

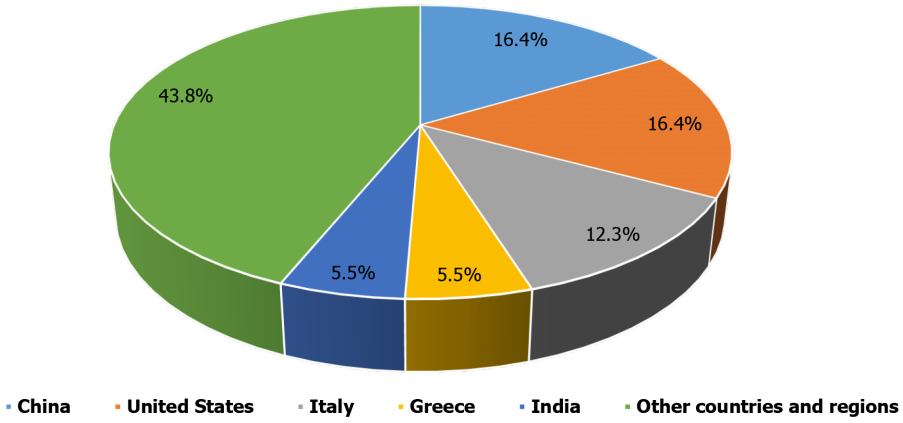


Figure 3 Country distribution of Editorial Board members of the *World Journal of Clinical Oncology*.

overcome: (1) The sources of manuscripts are lacking, especially for high-quality original and review manuscripts; (2) There are not enough experts to participate in peer review for each manuscript, leading to a low rate of final submission of peer-review reports; and (3) Due to the insufficient number of Editorial Board members and the uneven distribution of regions and disciplines, some manuscripts progress through the review process too slowly. In 2021, it is imperative to solve these problems.

First, we need to establish a connection with high-level oncology experts and promote their submissions of high-quality original or review articles. Second, the number of Editorial Board members must be increased with an eye towards balancing the distribution of experts in different areas and disciplines. Third, we need to further optimize the entire review-to-publication process with the aims of shortening the time of peer review and providing high-quality and efficient peer-review reports. Finally, we need to continue our latest company-wide strategy of sending the journal's latest published articles to target readers *via* email; this is an effective activity by which we will increase the exposure rate and citation rate of these articles, thereby expanding the academic influence of our authors.

CONCLUSION

With the continued efforts and support of all internal and external contributors to the *WJCO*, we expect to make greater strides forward in 2021. For our part, we commit to working diligently alongside all of you to raise the academic rank of *WJCO* in 2021 and merit inclusion in the Science Citation Index Expanded as soon as possible. We appreciate that the achievement of these goals is reliant upon the continuous support and submissions from authors worldwide and the dedicated efforts and expertise by our invited reviewers, many who also serve on our Editorial Board. We are also open to any suggestions that could improve the *WJCO*'s operation and publication. Please feel free to contact us at editorialoffice@wjgnet.com with any questions on your submission or suggestions.

Once again, on behalf of the *WJCO*'s Editorial Office, we wish you and your families the best for the New Year.

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OPINION REVIEW

Pancreatic cancer in the era of COVID-19 pandemic: Which one is the lesser of two evils?

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Abstract

Pancreatic adenocarcinoma remains one of the deadliest malignancies affecting the older population. We are experiencing a paradigm shift in the treatment of pancreatic cancer in the era of coronavirus disease 2019 (COVID-19) pandemic. Utilizing neoadjuvant treatment and further conducting a safe surgery while protecting patients in a controlled environment can improve oncological outcomes. On the other hand, an optimal oncologic procedure performed in a hazardous setting could shorten patient survival if recovery is complicated by COVID-19 infection. We believe that oncological treatment protocols must adapt to this new health threat, and pancreatic cancer is not unique in this regard. Although survival may not be as optimistic as most other malignancies, as caregivers and researchers, we are committed to innovating and reshaping the treatment algorithms to minimize morbidity and maximize survival as caregivers and researchers.

Key Words: Pancreatic cancer; COVID-19; Medical oncology; Radiation; Neoadjuvant therapy; Pancreatic adenocarcinoma

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Core Tip: Pancreatic adenocarcinoma remains one of the deadliest malignancies affecting the older population; Oncological treatment protocols must adapt to the coronavirus disease 2019 era; Neoadjuvant treatment and further conducting a safe surgery while protecting patients in a controlled environment can improve oncological outcomes.

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INTRODUCTION

Pancreatic adenocarcinoma remains one of the deadliest malignancies affecting the older population. Surgery has been the mainstay of treatment and the only chance for complete oncological cure. Despite the advancements in chemotherapy and radiation oncology, only 20% of cases make it to surgery[1]. As the coronavirus disease 2019 (COVID-19) is swiftly evolving, we are at war on two fronts in all patients diagnosed with recent malignancy. These patients are among the most vulnerable. As of the third week of December 2020, there are more than 79 Million cases and more than 1.7 Million deaths Worldwide. Despite the mitigating measures implemented by the local authorities, the federal government and health organizations across the nation, the disease portends severe morbidity and mortality[2].

According to the American Cancer Society, approximately 57600 new pancreatic cancer cases will be diagnosed in the United Sates in 2020. Due to the aggressive biology of pancreatic cancer, robust radiological and pathological diagnosis cannot be hastened enough. Tele-medicine can lessen the impact of restrictive social distancing on multi-disciplinary patient counseling, although it does not resolve the need for infusion visits, radiation simulation and treatment sessions, hospital admissions, imaging studies, laboratory visits and maintaining the minimal requirements for patients involved in clinical trials. Many challenges still persist and include: (1) The role of diagnostic and interventional endoscopy in the setting of low-yield viral testing, unknown dissemination and uncertain hazard for health workers; (2) The scarcity of allocated healthcare resources to meet the supply chain demands at the pandemic epicenters, as well as, implementing social distancing behaviors limiting the accessibility of patients to on-site cancer treatments; (3) The morbidity and mortality emerging from the combined effect of chemotherapy-associated immunosuppression and COVID-19 adverse outcomes; (4) The high mortality of COVID-19 infection on patients in the pre- and post-operative period[3]; and (5) The mental toll on both the patient and family in times of an already overwhelming cancer diagnosis. This is particularly difficult in the COVID-19 era as family members are unable to be with patients in the office setting, before surgery, and during the postoperative recovery. Travel restrictions and visitor limitations intensify this effect.

Caregivers involved in any aspects of cancer patients' care should be absolutely committed to providing evidence-based and peer-reviewed oncological care. It is a mandatory requirement for National Cancer Institute (NCI)- designated cancer centers to review each cancer case at a multi-disciplinary conference as a provision of standard of cancer care. Although these meetings have largely been retooled in the era of COVID-19 to occur both virtually and remotely to assure staff distancing, their value in supporting real-time multidisciplinary communication among clinicians has become increasingly critical.

As cancer care providers, we carry out our responsibilities to maintain the optimal care for pancreatic cancer patients who suffer from poor prognosis at baseline. The limited benefit of treating metastatic pancreatic cancer and the risk of enhancing morbidity and accelerating mortality should be considered on a case-by case basis. In this article, we illustrate our team's approach to the management of pancreatic cancer at an NCI-designated comprehensive cancer center in the era of the COVID-19 pandemic. As these practices are still evolving, we anticipate more innovative interventions to improve the oncological care of patients with pancreatic cancer and to decrease the risk of COVID-19 dissemination among this vulnerable population.

Table 1 demonstrates the evolution of pancreatic cancer staging and workup during the COVID-19 outbreak at our institute.

COVID-19 AND THE RESECTABILITY OF PANCREATIC CANCER

Our surgical and medical management of pancreatic cancer revolves around the current classification of this disease into a resectable, borderline resectable (BLR) and locally advanced (LA) disease. Resectable disease is defined as no tumor contact with the celiac axis (CA), superior mesenteric artery (SMA) and common hepatic artery (CHA), and no or ≤ 180° contact with the superior mesenteric vein (SMV) and portal vein (PV) without venous contour irregularity. LA disease is defined as tumor contact with SMA or CA > 180°, or un-reconstructible SMV/PV due to tumor invasion and occlusion. BLR disease is any tumor and arterial/venous relationship in between of the above[4].

The additional morbidity and mortality of a superimposed COVID-19 infection must be considered in the decision making for these patients. The war at these two fronts is relentless and any strategic planning should take into account the patient as a whole rather than these two high-risk diseases independently. Therefore, in high risk patients and on a case-by-case basis, we consider any degree of abutment of the SMV and PV as an opportunity to introduce BLR treatment approaches rather than surgery as a first-line approach.

UTILITY OF NEOADJUVANT THERAPY IN PANCREATIC CANCER

The use of neoadjuvant therapy in the treatment of those with pancreatic cancer is not novel, and there has been a growing body of literature to support this. This approach's foundation has been extrapolated from the adjuvant therapy data, which was well established from many clinical trials such as the ESPAC-1 and CONKO-001 trials[5-7]. The theoretical benefit of neoadjuvant therapy is to treat micro-metastatic/occult disease, down-stage the tumor from BLR or LA to resectable, and measure tumor chemosensitivity[8]. Another advantage is that patients are able to receive more of their intended treatments upfront, addressing the risk of low adjuvant treatment accruals following pancreatoduodenectomy for resectable pancreatic cancer[9].

Two specific chemotherapy regimens have gained enthusiasm in the oncologic practice: 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) and gemcitabine/Nab-paclitaxel (Gem/NabP). The use of FOLFIRINOX has been proven in the metastatic setting, with the results from a phase III ACCORD-11/PRODIGE-4 trial demonstrating improved overall survival over single agent gemcitabine[10]. The phase III MPACT trial showed improved survival with Gem/NabP in the metastatic setting as well, when comparing to gemcitabine alone[11]. While there are mounting data to support its use in those with BLR and LA pancreatic cancer, there are also emerging data supporting its use in those with resectable disease. The randomized phase III PREOPANC trial, addressing the role of preoperative gemcitabine-based chemoradiotherapy *vs* immediate surgery for resectable and BLR pancreatic cancer in 16 centers, was recently published[12]. Although this study reported no significant overall survival benefit, it demonstrated improved R0 resection, disease-free survival and locoregional failure-free interval in patients who received preoperative chemoradiotherapy. The occurrence of serious adverse events was 52% *vs* 41%, respectively (P = 0.096)[12]. The benefit of preoperative FOLFIRINOX-based chemotherapy is currently being investigated by the PREOPANC-2, NorPACT-1 and PANACHE01-PRODIGE48 trials.

While there is an ongoing debate on how best to sequence the necessary components of pancreas cancer treatment in these patients, the rationale for neoadjuvant chemotherapy, including that advantage of being able to gauge response to systemic treatment, to potentially downstage higher T stage and node-positive tumors, to treat micro-metastatic disease early, and to promote maximal completion of all systemic treatments, all serve as important advantages to this approach *vs* an approach where newly diagnosed patients are taken immediately to surgery. However, it is important to balance the potential advantages of neoadjuvant chemotherapy in pancreatic cancer with the risks associated with immunosuppressive chemotherapy in the COVID-19 era. The American Society of Clinical Oncology has published recommendations to guide medical oncologists in the current pandemic, although none of the recommended practice adaptions is particularly relevant to

Table 1 Evolution of pancreatic cancer staging and workup in the era of coronavirus disease 2019

Component of care	Pre COVID-19	Risk group	Post COVID-19
Multidisciplinary evaluation	Appointments with medical, radiation, surgical oncologists and gastroenterologists	Patients and health care providers	Tele-medicine
Tumor board Conference	On-site meetings	Excess contact of involved caregivers	Virtual meeting
Diagnostic Methods	Sectional imaging, endoscopic interventions and laboratory visits	Excess contact of health care providers and patients	Reduce diagnostic interventions to minimum needed, avoidance of endoscopic biopsy
Surgical staging	Pre-resection laparoscopic staging	Patients, surgeons and operative room staff	Substitute by imaging
Genetic evaluation	Inperson counseling/testing	Patient, family and counselor	Tele-counseling

COVID-19: Coronavirus disease 2019.

patients with a newly diagnosed resectable pancreatic cancer. The American Society of Breast Surgeons has advocated for the use of neoadjuvant therapy among patients with high risk tumors like triple negative breast cancer, as well as the potential for less frequent dosing schedules to reduce infusion room time and therapy visits[13].

Radiation or chemoradiation may be recommended in cases where surgery is being delayed due to COVID-19 concerns. In these cases, it is reasonable to pursue a treatment approach that has been shown to limit local progression rates in an effort to delay surgery. Many radiation oncology departments have been able to limit exposure risks with interventions such reduction in the number of patients coming in for treatment, social distancing in waiting areas, increased time between scheduled patients, more frequent cleaning of high touch surfaces, increased use of masks and in some settings gloves, and strict hand hygiene measures. To further minimize exposure, consideration also needs to be given to the number of visits that are necessary to treat a patient. While there are data supporting longer courses of daily radiation for 5-6 wk, the value of these longer schedules with conventional fractionation should be weighed against the exposure risks of up to 30 visits. By utilizing advanced techniques such as intensity modulated radiation therapy, image-guided radiation therapy, stereotactic body radiotherapy and magnetic resonance imaging-guided adaptive radiation therapy, it may be possible to increase the fraction size and significantly shorten the schedule. Of course, one complicating factor of this move to more precise radiation techniques is that some require placement of a fiducial marker *via* an endoscopic procedure, which may increase the exposure risk to both patients and staff. All of these factors are important to weigh for each individual patient. Table 2 summarizes the risks and benefits of immediate resection *vs* neoadjuvant treatment followed by resection for resectable pancreatic cancer in the era of COVID-19.

TIMING OF SURGERY

Elective surgery confers a high mortality during the incubation period of COVID-19 infection[3]. This has been attributed to the immune dyscrasia associated with the stress of invasive procedures which culminated into acceleration of symptomatic presentation[14]. Although few studies are available, the perioperative mortality rate has been reported to be as low as 21% in Wuhan, China (47% after intensive care unit admission) and as high as 75% in Iran[3,15]. This is more pronounced in the older population who are typically the affected group in pancreatic cancer[3]. Acute respiratory distress syndrome and acute cardiomyopathy resulting into cardiac shock and fatal arrhythmia were the most common causes of death[3]. The contemporary accepted perioperative mortality rate for pancreaticoduodenectomy at high-volume cancer centers is 2%-4%[16].

The main challenge is considering operative intervention on those who have completed neoadjuvant chemoradiation during the peak of COVID-19 cases. We are evaluating, on a case-by-case basis, the potential of delaying operative intervention for a few weeks until the peak of the curve is flattened in the southeastern Pennsylvania region. Patients who are currently tolerating chemotherapy are being counseled to continue their current regimen until the postulated date of resection. On the other

Table 2 Risks and benefits of immediate resection versus neoadjuvant therapy followed by resection for resectable pancreatic cancer in the era of coronavirus disease 2019

	Immediate pancreatic resection	Neoadjuvant chemoradiation
Patients	(1) Increased perioperative morbidity and mortality; and (2) Exposure risk to COVID-19 with excess contacts	(1) Better completion rates; (2) Avoidance of surgery in those chemo-resistant and rapidly progressing; (3) Less caregivers can be in direct contact with the patient; and (4) No data is available weighing the gravity of immunosuppressive chemotherapy versus immediate surgery on morbidity and mortality in the era of COVID-19
Health care providers	(1) Exposure risk of surgical personnel and postoperative caregivers; and (2) Most hospitals are still in the process of implementing local mitigating measures in the surgical settings	(1) Exposure risk of medical, radiation oncology, and infusion center personnel; and (2) Delay of surgery until better mitigating protocols are implemented by local surgical operations

COVID-19: Coronavirus disease 2019.

hand, we must keep in mind that delaying surgery following neoadjuvant radiation therapy can have undesirable consequences. Radiation induced fibrosis can distort anatomical planes and increases surgical difficulty. A recent study conducted at our institute on 44 patients who received neoadjuvant chemoradiation demonstrated that the optimal time to perform pancreatic resection is 6-8 wk after completion of radiotherapy.

PREOPERATIVE TESTING AND PREPARATION

All pancreatic cancer cases are scheduled as open procedures to prevent the aerosol exposure of coronavirus suggested by other studies on similar pathogens[17]. The vast majority of the initially available and experimental COVID-19 tests were believed to act as "rule in" rather than "rule out" tests with relatively high false negative rate[18]. Also current known cases of COVID-19 might actually be an underestimation of the definite spread of the virus in the community. Hence, all patients presenting to our operative facility are suspected to incubate COVID-19 until we have more accurate tests available. Despite their current limited distribution and scarcity, many effective vaccines have the potential benefit of protecting and prepare this vulnerable population for their medical and surgical treatment.

Operative room, staffing and recovery unit precautions are implemented according to the continuously updated American College of Surgeons and Center for Disease Control and Prevention recommendations. At our institute, we review the weekly oncological procedures being performed on the preceding Thursday during a departmental meeting. This approach ensures the validity of performing such procedure during the current crisis. Procedures that deemed not necessary or can be postponed without compromising the oncological outcomes are followed closely for future operative considerations. Due to the scarcity of resources, the usage of personal protective equipment and the sterilization process of N95 masks are monitored very tightly by the surgical operations. This ensures maximal protection, minimal wasting and effective involvement of the least number of operative personnel needed to conduct an efficient and safe operation.

INSTITUTIONAL MEASURES IMPLEMENTED TO MINIMIZE COVID-19 DISSEMINATION

The course of COVID-19 is long and associated with high transmissibility from asymptomatic patients going through a prolonged incubation period[19]. As a designated comprehensive cancer center, many measures were implemented to protect our vulnerable patients. All employees have their temperature taken upon their arrival to the health campus. Employees with temperatures below 100 °F are given surgical masks to be worn all the time. Those whose temperature is 100 °F or higher are asked to put on masks and self-isolate at home while waiting for occupational health evaluation.

Our visitor policy has been revised to prohibit inpatient visits on campus unless necessary in the setting of end-of-life discussions. Patients who present for surgery or on-site treatments are not allowed to be accompanied by visitors and subsequent

transportations are provided by the hospital. Tele-medicine technology has begun to replace regular outpatient visits; these include primarily consultative visits and any visits that do not require on-site treatments or radiological investigations. Quarantine units have been arranged and protocols have been created in the case of a local outbreak. Inpatients who test positive for COVID-19 infection are isolated from other patients in a separate facility and being cared for by a specialized dedicated team.

CONCLUSION

Which is the lesser of two evils is an open-ended question with no true answer. Both pancreatic cancer and COVID-19 are diseases that require utilization of substantial healthcare and human resources. Therefore, we are experiencing a paradigm shift in the multidisciplinary management of pancreatic cancer that must balance the risks of the disease and the risks of treating this deadly disease while the specter of COVID-19 Looms. Utilizing neoadjuvant treatment and further conducting a safe surgery while protecting patients in a controlled environment can improve oncological outcomes, but may also permit delay of high risk, resource heavy pancreatectomy while hospitals funnel valuable resources to address the surge of COVID-19 cases in their communities. Even an optimal oncologic procedure performed in a hazardous setting could shorten patient survival if recovery is complicated by factors such as COVID-19 infection.

The era of COVID-19 is the new normal, for now. We cannot make the mistake of under-estimating the severity and potential duration and long-term impact of this crisis on cancer care and public health. Therapeutic measures are being tested to fight the virus, but there are no guarantees for when these may allow a safe resumption of everyday living as well as previously adopted practices for managing cancer patients. Oncological treatment protocols must adapt to this new health threat, and pancreatic cancer is not unique in this regard. Although long-term survival may not be as optimistic as most other malignancies, as caregivers and researchers, we are committed to innovate and reshape treatment algorithms to minimize morbidity and maximize the chances of survival for our sickest patients.

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EVIDENCE REVIEW

New frontiers in focal therapy for prostate cancer: Prostate-specific membrane antigen positron emission tomography/magnetic resonance imaging

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Abstract

Imaging has a central role in the context of focal therapy (FT) for prostate cancer (PCa). Prostate-specific membrane antigen (PSMA) positron emission tomography/magnetic resonance imaging (PET/MRI) is a novel imaging modality that combines the morpho-functional information of MRI with the molecular characterization of PET. Some papers reported the potential advantages of PSMA PET/MRI in different clinical scenarios. Limited evidence on PSMA PET/MRI is available in the setting of FT. PSMA PET/MRI can be an effective imaging modality for detecting primary PCa and seems to provide accurate local staging of primary PCa. PSMA PET/MRI also shows high performance for restaging and detecting tumor recurrence. The higher soft-tissue contrast and the reduction of ionizing radiation are the main advantages reported in the literature compared to PET/computed tomography. PSMA PET/MRI could represent a turning point in the management of patients with PCa in the context of FT. Further studies are needed to confirm its applications in this specific clinical

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setting.

Key Words: Prostate-specific membrane antigen; Positron emission tomography/magnetic resonance imaging; Prostate cancer; Focal therapy; High-intensity focused ultrasound; Cryotherapy

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Core Tip: Prostate-specific membrane antigen positron emission tomography/magnetic resonance imaging can be an effective imaging modality for detecting primary and recurrent prostate cancer, besides it seems to provide accurate local staging and restaging. Multiparametric magnetic resonance imaging is considered the standard imaging modality in the context of focal therapy; however, the diagnostic performance of prostate-specific membrane antigen positron emission tomography/magnetic resonance imaging make it an excellent candidate as a technique of choice in this setting.

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INTRODUCTION

Prostate-specific membrane antigen (PSMA) is a membrane protein that is overexpressed in the vast majority of prostate cancer (PCa)[1]. PSMA can be used as a positron emission tomography (PET) target through specific ligands labeled with radioisotopes. PSMA PET/computed tomography (CT) is an established imaging technique for the evaluation of biochemical recurrence (BCR) of PCa[2] and showed possible applications also for tumor detection, staging, treatment planning, and assessment of response to therapy[3-6]. PSMA PET/magnetic resonance imaging (MRI) is a hybrid imaging technique that combines the morphological information of MRI with the molecular data of PET. MRI provides excellent anatomical characterization and soft tissue contrast, whereas PSMA PET offers a reliable molecular characterization of the tumor[7]. The first applications of PSMA PET/MRI in the PCa setting were described in 2013[8]; since that time, several papers reported the potential applications of this imaging modality in different clinical scenarios[9,10]. Multiparametric (mp) MRI is considered the standard imaging modality for tumor detection, local staging and follow-up in the focal therapy (FT) setting[11]; however, the demonstration of several intrinsic limitations of this technique highlighted the need to investigate alternative imaging modalities, including PSMA PET/CT and PSMA PET/MRI[12,13].

PRIMARY PROSTATE CANCER DETECTION AND STAGING WITH PROSTATE-SPECIFIC MEMBRANE ANTIGEN POSITRON EMISSION TOMOGRAPHY / MAGNETIC RESONANCE IMAGING

Eiber *et al*[14] found that patient-based sensitivity for the tumor detection of mpMRI, PSMA PET, and PSMA PET/MRI was 66%, 92%, and 98%, respectively. Patient-based sensitivity of PSMA PET and PSMA PET/MRI statistically significantly outperformed mpMRI (P = 0.001 and P < 0.001), while no significant difference was observed between PSMA PET imaging and PSMA PET/MRI (P = 0.250). With cut-off scores of 3 for mpMRI and 4 for PSMA PET and PSMA PET/MRI, lesion-based sensitivity and specificity of PSMA PET/MRI were 76% and 97% respectively, while mpMRI and PSMA PET showed less sensitivity (58% and 64%) and specificity (82% and 94%). A concordance subanalysis revealed that both mpMRI and PSMA PET were able to

identify most tumors; however, each technique detected tumor-involved areas that were negative in the other modality, this contributed to the observed superiority of PSMA PET/MRI. No significant correlation was found between quantitative PET parameters and Gleason score (GS). Al-Bayati *et al*[15] found that lesion-based sensitivity for the tumor detection of mpMRI, PSMA PET, and PSMA PET/MRI was 59%, 81%, and 88%, respectively. PSMA PET and PSMA PET/MRI had a significantly higher sensitivity than mpMRI (P = 0.03 and P = 0.003), while they did not show a significant difference in between them (P = 0.5). The lesion-based specificity of mpMRI, PSMA PET, and PSMA PET/MRI was 66%, 100%, and 100%, respectively. PSMA PET and PSMA PET/MRI rated 4 and 6 Lesions as equivocal (5-point Likert scale 3), while mpMRI classified 15 Lesions as indeterminate (PI-RADS 3). In a considerable proportion of equivocal results with mpMRI, PSMA PET led to a correct shift towards higher malignancy suspicion. Hicks *et al*[16] showed the improved region-specific sensitivity for the tumor detection of PSMA PET/MRI compared to mpMRI. Besides, the authors found a significant correlation between tumor maximum standardized uptake value and GS. Park *et al*[17] concluded that PSMA PET/MRI offers incremental value over a dedicated mpMRI for preoperative PCa localization and staging. Sugawara *et al*[18] reported a higher accuracy for primary tumor diagnosis of PSMA PET/MRI compared to mpMRI, PSMA PET or clinical factors alone (*i.e.*, digital rectal examination and PSA), and the combination of PSMA PET/MRI with clinical profile improved the characterization of lesions; besides, the authors reported the significant association of maximum standardized uptake value with GS. Freitag *et al*[19] concluded that the lymph node (LN) and bone metastases were accurately and reliably depicted by PSMA PET/MRI with very low discordance compared to PSMA PET/CT. Both PET techniques were able to identify metastases in normal-sized LN (71.9%). Visibility of LN was significantly higher with MRI compared to CT using T1-w CE (P = 0.013), T2-w fat-saturated (P < 0.0001), and DWI (P < 0.0001) sequences. Two PSMA PET-positive bone metastases could not be confirmed morphologically using CT, but it was possible with MRI.

Maurer *et al*[20] found that PSMA PET/MRI and PSMA PET/CT for LN staging performed significantly superior to morphological imaging alone (CT or MRI) on a patient- (P = 0.002) and template-based analysis (P < 0.001). In contrast, no substantial difference between CT and MRI as morphological imaging components of PSMA PET was recorded. Thalgott *et al*[21] assessed the diagnostic potential of PSMA PET/MRI compared to preoperative staging nomograms and concluded that PSMA PET/MRI and clinical nomograms performed equally well to determine the clinical stage; besides, PSMA PET provided additional anatomical information useful for therapeutic planning. Grubmüller *et al*[22] reported the correct identification of PCa with PSMA PET/MRI in 122 patients (97.5%). The accuracy of T-and LN-staging in 80 patients was 82.5% (95% Confidence interval: 73%-90%) and 93% (95% Confidence interval: 84%-98%), respectively. Noteworthy, PSMA PET/MRI changed the therapeutic strategy in 28.7% of the patients. Muehlematter *et al*[23] compared the diagnostic accuracy of mpMRI and PSMA PET/MRI for the detection of extracapsular extension (ECE) and seminal vesicle infiltration. PSMA PET/MRI showed lower region-specific sensitivity for detection of ECE (90% *vs* 94%; P = 0.007) and seminal vesicle infiltration (94% *vs* 98%; P = 0.001), while patient-specific sensitivity for the detection of ECE was higher with PSMA PET/MRI (69% *vs* 46%; P = 0.04). No other significant differences were found. Ferraro *et al*[24] found that the patient-based sensitivity, specificity, PPV, and NPV for the LN metastases detection of PSMA PET/MRI were 58%, 98%, 88%, and 90%, respectively. The model combining PSA, Gleason score, and PSMA PET visual analysis of LN showed a tendency to improve patient selection for LN dissection over the currently used clinical nomograms. Kaufmann *et al*[25] investigated the accuracy for T-and LN-staging of PSMA PET/MRI, surprisingly reporting discordant results compared to the previous ones. The authors observed similar overall PCa detection between mpMRI and PET/MRI (both ^{11}C-choline and ^{68}Ga-PSMA). mpMRI was found to be more accurate than PET for T-staging. In particular, PET underestimated the local tumor extent and no additional value for PET concerning the primary tumor extend was found. mpMRI showed no inferiority compared to PET/MRI in N-staging, and the author reported the limitation of PET/MRI in detecting small LN metastases independent of the radiotracer used.

RECURRENT PROSTATE CANCER DETECTION AND RESTAGING WITH PROSTATE-SPECIFIC MEMBRANE ANTIGEN POSITRON EMISSION TOMOGRAPHY / MAGNETIC RESONANCE IMAGING

Afshar-Oromieh *et al*[26] compared PSMA PET/CT and PSMA PET/MRI in BCR patients. The authors concluded that, outside the "halo artifacts" around the bladder and at the level of the kidneys, PSMA PET/MRI was more accurate and enabled a subjectively easier evaluation of the images than PSMA PET/CT, allowing to clarify unclear findings on PSMA PET/CT. Freitag *et al*[27] reported that 93 (78.2%) BCR patients had PSMA-positive lesions. Eighteen (15.1%) subjects had local recurrences in PET/MRI, while only 9 (7.6%) in PET/CT (P = 0.004). Bladder-to-local recurrence distance was identified as a statistically significant predictor of PSMA PET-positivity (P = 0.028), contrary to local recurrence size (P = 0.84). Hope *et al*[28] found that with PSMA PET/MRI and PSMA PET/CT the disease was detected in 103 (82%) BCR patients and major changes in the management was preferred in 67 (53.2%) cases; however, no sub-analysis was performed to compare the two PET techniques. Lütje *et al*[29] showed that in 14 (29.2%) patients neither PSMA PET/CT nor PSMA PET/MRI found lesions and 9 (19.7%) were excluded due to artifacts around the bladder. In the other 25 (52.1%) subjects, PSMA PET/MRI *vs* PSMA PET/CT identified 14 *vs* 9 recurrences in the prostate bed, 23 *vs* 20 PET-positive lymph nodes, and 4 *vs* 4 PSMA PET-positive bone lesions, respectively. The higher detection of tumor recurrences in the prostate bed of PSMA PET/MRI was attributed to the superior soft-tissue contrast of MRI component. Schiller *et al*[30] found that lesions suspicious for PCa were detected in 27/31 cases (87.1%) with PSMA PET/MRI or PSMA PET/CT compared to negative CT/MRI. Furthermore, 14 patients (45.2%) had a changed staging result with PSMA PET compared to CT/MRI. Grubmüller *et al*[31] showed that PSMA-positive lesions were found in 100 (85.5%) BCR patients with PET/CT or PET/MRI, reporting a detection rate of 65% for PSA levels of 0.2-0.5 ng/mL. PSMA PET detected lesions in 67 (57.3%) subjects who had no suspicious with MRI or CT and changed therapeutic management in 74.6% of them. The authors did not compare the performance of two PET modalities. Kranzbühler *et al*[32] reported positive PSMA PET/MRI in 44 (78.6%) BCR patients. Suspicious lesions were detected in 44.4%, 72.7%, 80%, 95.2% of subjects with PSA levels of < 0.2, 0.2-0.5, 0.5-2.0, > 2.0 ng/mL respectively. The detection rate of MRI was significantly lower than PSMA PET/MRI (24%), while the overall detection rate of PSMA PET was comparable with PSMA PET/MRI (76%). The high detection rate (54.5%) for recurrent PCa, even at low PSA levels (< 0.5 ng/mL), were confirmed in a subsequent study of the same group[33]. Burger *et al*[34] investigated PSMA PET/MRI's performance for the localization of disease recurrence in patients undergoing high-intensity focused ultrasound with proven significant PCa on transperineal template biopsy not detected with mpMRI. It is necessary to emphasize that this is the only paper available in literature analyzing PSMA PET/MRI in the specific context of FT. PSMA PET was positive in 6 (60%) patients. No false-positive lesions were reported. All negative subjects had GS 3 + 4 disease, while all lesions with GS 4 + 3 or higher were detected. The quadrant-based sensitivity, specificity, PPV, and NPV of PET were 55%, 100%, 100%, and 85%, respectively. Abufaraj *et al*[35] assess the accuracy of PSMA PET/CT or PSMA PET/MRI LN staging in patients with BCR after RP undergoing LN dissection. Patient-based sensitivity was 100%. At regional analysis, sensitivity of PET ranged from 72% to 100%, specificity from 96% to 100%, PPV from 95% to 100%, NPV from 93% to 100%, diagnostic accuracy from 95% to 98%. No differences in diagnostic performance were found between PSMA PET/CT and PSMA PET/MRI. The PPV in patients with a PSA level ≥ 1.4 ng/mL was almost always 100% in all regions and subregions except the presacral region (93%). Guberina *et al*[36] reported that tumor recurrence was localized in 62 (66.7%) BCR patients based on combined PSMA PET/CT and PSMA PET/MRI reading. The sensitivity of PSMA PET/MRI and PSMA PET/CT was 98.8 % and 93.2%, respectively. PSMA PET/MRI detected 148 out of 150 Lesions described in PSMA PET/CT (missing two LN lesions) and other 11 Lesions (5 LN lesions and 6 Local recurrences). A significant difference (P = 0.031) between PSMA PET/CT and PSMA PET/MRI for local recurrence diagnosis was found.

SUMMARY OF EVIDENCE

PSMA PET/MRI may be an effective imaging modality to detect primary PCa,

showing a higher accuracy compared to mpMRI alone[14-16]. It provides accurate local staging of primary PCa; however, there are contradictory results in this setting when its reliability is compared to other imaging modalities[20,24,25]. PSMA PET/MRI also shows high performance for restaging and detecting tumor recurrence, but its superiority over PSMA PET/CT has not yet been clearly demonstrated[8,31,35] (Table 1). PSMA PET/MRI seems to favorably integrate the current clinical nomograms[21,24]. Artifacts can reduce the diagnostic performance of PSMA PET around the bladder and kidneys[26,27]. The reduction of ionizing radiation and the higher soft-tissue contrast and the main advantages reported in the literature compared to PSMA PET/CT, while the long duration, the high cost, the poor standardization of the technique, and the low availability are some relevant limitations[37,38].

CONCLUSION

The ideal imaging technique for prostate cancer patients in the focal therapy setting is not yet available but encouraging data regarding PSMA PET/MRI are emerging from the literature.

PSMA PET/MRI could represent a turning point in the management of patients with prostate cancer in the context of focal therapy; however, well-designed studies are needed to clarify the role of PSMA PET/MRI in this specific clinical setting.

Table 1 Diagnostic performance of imaging techniques in the focal therapy setting

	mpMRI	PSMA PET/CT	PSMA PET/MRI
Primary tumor detection	Intermediate	Intermediate/High	High
Loco-regional staging	Intermediate/High	Intermediate/High	Intermediate/High
Tumor recurrence detection	Intermediate	Intermediate/High	Intermediate/High

FT: Focal therapy; PSMA: Prostate-specific membrane antigen; PET: Positron emission tomography; CT: Computed tomography; MRI: Magnetic resonance imaging; mp: Multiparametric.

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REVIEW

Chronic myeloid leukemia-from the Philadelphia chromosome to specific target drugs: A literature review

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Abstract

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm and was the first neoplastic disease associated with a well-defined genotypic anomaly — the presence of the Philadelphia chromosome. The advances in cytogenetic and molecular assays are of great importance to the diagnosis, prognosis, treatment, and monitoring of CML. The discovery of the *breakpoint cluster region (BCR)-Abelson murine leukemia (ABL) 1* fusion oncogene has revolutionized the treatment of CML patients by allowing the development of targeted drugs that inhibit the tyrosine kinase activity of the BCR-ABL oncoprotein. Tyrosine kinase inhibitors (known as TKIs) are the standard therapy for CML and greatly increase the survival rates, despite adverse effects and the odds of residual disease after discontinuation of treatment. As therapeutic alternatives, the subsequent TKIs lead to faster and deeper molecular remissions; however, with the emergence of resistance to these drugs, immunotherapy appears as an alternative, which may have a cure potential in these patients. Against this background, this article aims at providing an overview on CML clinical management and a summary on the main targeted drugs available in that context.

Key Words: Chronic myeloid leukemia; Breakpoint cluster region-Abelson murine

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Core Tip: Chronic myeloid leukemia is a clonal hematopoietic stem cell disorder with a well understood pathogenesis. In this sense, the research is directed towards new therapeutic alternatives and understanding of immunobiology, considering the growing resistance to standard therapy with tyrosine kinase inhibitors. This article aims to summarize the clinical approach and emerging therapeutic alternatives, considering the adverse effects of new drugs, transplants and immunotherapy.

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INTRODUCTION

Chronic myeloid leukemia (CML) is a malignant myeloproliferative disorder characterized by a clonal hematopoietic stem cell proliferation. CML was the first malignant disease related to a cytogenetic abnormality[1] and its pathogenesis has been extensively studied. The advances in this field allowed the development of targeted therapies with tyrosine kinase inhibitors (TKIs) with high rates of therapeutic success, increasing substantially patient survival and disease prevalence[2]. As for its incidence, CML affects about 0.7-1.0/100000 individuals per year and this rate has been stable over the last few years. With regard to the sociodemographic profile of CML patients, there is a slightly higher predominance among men, and the diagnosis usually occurs around the sixth or seventh decade of life[3].

CML is characterized by the presence of the Philadelphia chromosome, which is the result of a balanced reciprocal translocation between the long arms of 9 and 22 chromosomes [t (9; 22) (q34; q11)]. The fusion of the *Abelson murine leukemia* (*ABL*) gene on chromosome 9 with the *breakpoint cluster region (BCR)* gene on chromosome 22 results in the *BCR-ABL1* fusion gene, which encodes the BCR-ABL oncoprotein[4]. This protein is a persistently active tyrosine kinase that promotes unrestricted replication, inadequate differentiation, and resistance to apoptosis[4,5]. The continuous proliferation of these stem cells with a high capacity for differentiation favors the appearance of additional mutations that can provide resistance to standard treatment representing a negative impact on prognosis[6].

Despite the high success rate in treatments with TKIs, the emergence of resistance to TKIs has led to the development of new drugs and immunotherapy has been considered as an alternative to these patients, aiming to reduce disease recurrence and chronic use of medication[7].

CML: A BRIEF HISTORIC REVIEW

CML was the first leukemia discovered, being described around 1840 by David Craigie, John Hughes Bennet, and Rudolph Virchow through autopsies on individuals who had presented with very similar findings, such as hepatosplenomegaly, fever, leukocytosis[8], and an unusual blood appearance and consistency, described by Alfred Velpeau in the 19th century as "thick blood"[9]. There were several interpretations concerning such blood aspects throughout the years. Initially, some specialists attributed the thick blood to the presence of pus due to some infectious process[9,10]; however, such condition was not diagnosable even with autopsy. The explanation that

refuted the purulent blood theory came from Alfred Donné. He detected a large amount of white blood cells, resulting from an interruption in the hematopoietic cells maturation[9]. In 1845, John Hughes Bennett hypothesized that an infection generates what he called leukocytemia (white cell blood), due to the large amount of white blood cells found during the autopsy, and Virchow, in his turn, inferred that the disease is caused by hematopoietic changes, coining the term "weisses blut"-white blood (Leukemia - leukamie in German)[8,11].

In 1960, David Hungerford and Peter Nowell, two cytogenetics scholars, joined to determine if leukemia was linked to specific chromosomal abnormalities[12]. It was the first time that an association between an oncological disease and a chromosomal abnormality was established[13]. They identified the presence of a minute chromosome in two CML patients, which they called the "Philadelphia chromosome" (Ph) and, later, Janet Rowley refined this discovery by proving in 1973 that it was a balanced reciprocal translocation between the long arms on chromosomes 9 and 22: t (9; 22) (q34; q11)[14]. Currently, it is known that the Ph chromosome is not restricted to CML, and it can be found, especially in its p190 isoform, in individuals with acute lymphoblastic leukemia (ALL)[15]. However, the identification of this chromosome remains as an important cytogenetic marker of CML and its detection has implications for the diagnosis, prognosis, and treatment of the disease[16].

In addition to the genetic cause, in 1970, the possibility of leukemia also having a viral etiology was investigated based on the research by Herbert Abelson and Louise Rabstein, who identified the *ABL* gene in a murine virus and its physiological counterpart found in normal human cells[15,11]. From a retroviral infection of hematopoietic stem cells with *BCR-ABL1* P210 in mice, it was discovered that the fusion of a region of the *BCR* gene on chromosome 22 with another part of the *ABL1* gene on chromosome 9 results in balanced reciprocal translocation t (9; 22), demonstrating a disease induction similar to CML[17]. This fusion leads to the translation of proteins with high tyrosine kinase activity, culminating in uncontrolled signaling pathways and cell cycles of primitive leukocyte lines, which increases cell proliferation and inhibits apoptotic mechanisms[9,11,13,18].

PATHOGENESIS

Ph chromosome-related CML

The aforementioned mutation results in the production of oncoproteins[19]. In addition, epidemiological data describe that about 90%-98% of CML patients have this mutation [t (9;22) (q34; q11.2)], making the Ph chromosome the main pathological theory for the disease[20,21].

Notably, different proportions of that translocation may be reflected in different clinical presentations. These different phenotypes are caused by a variation in the BCRs, a process that can occur with the different proteins of the *ABL* gene: P210, P190, and P230. In this sense, it is noticed that the phenotype *BCR-ABL1* P210 is more related to CML (95%) than the phenotype *BCR-ABL1* P190, which is better associated with B-lymphoblastic leukemia/lymphoma (B-ALL/LBL), although it is found in about 1% of CML cases[22]. As for the P230 protein, it has been rarely described in the literature[21,23]. However, so far there is no clear elucidation of how these different phenotypes related to the *Ph* gene reflect different processes of leukemogenesis.

Regarding BCR-ABL-related neoplastic effects, there is an influence of this protein on several cell growth pathways, including RAS-RAF1-MEK-ERK, PI3K-AKT, and SFKs-STAT1-STAT5[24]. The Ph chromosome product revokes the need to stimulate the activation of these pathways, leading to uncontrolled and exacerbated cell growth and division[21]. Among the cellular effects generated by this neoplastic process, are the increase in the number of reactive oxygen species, breakage and damage in the repair of the DNA strand, lack of control of the typical cell cycle, dysregulation of cell adhesion, and inhibition of apoptosis and autophagy[25]. In addition, these changes make the initially mutated site more susceptible to further mutations, facilitating the progression of the disease[21]. In this sense, studies show that patients with CML mostly start from a single mutation, represented by the Ph chromosome, and, as far as the disease progresses, the rates of additional chromosomal abnormalities become higher (30%-70%)[26]. In this scenario, the natural history of the disease is slow to evolve during the chronic phase (CP), which, after the aforementioned events associated with the mutation site, gives rise to a phase of accelerated progression and a blast crisis (BC), also called blast phase[22].

The mechanisms that trigger the BC are not fully understood. This phase is

characterized by a marked proliferation of undifferentiated hematopoietic progenitors that can invade peripheral blood, and the role of the *BCR-ABL1* mutation appears to be less preponderant in this context. Moreover, higher rates of therapeutic failure with TKIs are observed among affected individuals and additional mutations are found in about 80% of these patients, such as double Ph chromosome, trisomy 8 or 19, isochromosome 17, and other mutations that may present alone or in combination. On the other hand, the presence of the active *BCR-ABL1* mutation seems to be necessary to trigger the BC, since it is uncommon in patients with good therapeutic response to TKIs; moreover, changes in cell division and differentiation promoted by this mutation may contribute to that process[27,28]. The main genetic abnormalities present in the stem cells associated with the BC are the higher expression of *MYC* proto-oncogene, inactivating mutations of p53, and increased β-catenin signaling, which play prominent roles on CML expansion and BC transformation[28].

Immunobiology of myeloid leukemia

The host immune system can be an important factor in disease progression and relapses by suppressing the residual leukemic cells that remain after molecular remission. In patients with CML, before treatment, an increased population of Treg cells and abnormal activation of the programmed death 1 (PD-1)/programmed death receptor ligand 1 (PD-L1) signaling is observed. The PD-L1 present in CML cells binds to PD-1 receptor on T cells, reducing its effector function[7,29]. The increased production of arginase I, common to several types of cancer and chronic diseases, by myeloid-derived suppressor cells results in the accelerated metabolism of L-arginine, an essential amino acid to the normal function of T cells. These factors result in a downregulation of the immune response[29,30].

The important role of the immune system in the prevention and control of CML is corroborated by the fact that, after allogeneic transplantation of pluripotent hematopoietic stem cells and lymphocyte infusion, the T cells eliminate the tumor by interacting with the tissue-restricted minor histocompatibility complex and with the leukemia-associated antigens. Furthermore, *BCR-ABL1*-positive healthy individuals are protected by immunosurveillance, although the mutation does not occur in the stem cells of these patients[31].

DIAGNOSIS

The diagnosis of CML is based on anamnesis, physical examination, and laboratory data, including cytogenetic and molecular tests. Through the association of these information, it is possible to identify the disease stage and to direct the most appropriate monitoring and treatment[32,33].

The diagnosis of CML usually occurs during routine medical appointments or blood tests in asymptomatic individuals[34]. About 30% to 50% of patients diagnosed with CML in the United States are asymptomatic[5,35]. The absence of symptoms is more common in the first of the three phases of the disease, the CP. Most CML diagnoses occur during this period, approximately 85%, with 40% being asymptomatic[2]. Right after the CP, most patients can progress to an accelerated phase (AP), and then the so-called BC can occur. However, approximately 20% of individuals progress directly from the CP to a BC, without any typical manifestation of the AP[5].

During the CP, the individual's immune system, if competent, maintains an asymptomatic status over a long period[36]. However, the CP can also manifest with symptoms such as anemia, splenomegaly, fatigue, weight loss, malaise, easy satiety and fullness, or pain in the upper left quadrant can occur. The less common manifestations during a CP are priapism, bleeding, thrombosis, retinal hemorrhage, and hepatomegaly[37]. Splenomegaly is the most common physical condition associated with CML, found in 50%-60% of the cases, whereas hepatomegaly develops in only 10%-20% of the patients[35,38].

During AP, the patients present with more severe symptoms that can include bone pain, skin infiltrate, lymphadenopathy, and worsening of the anemia[5,36]. Moreover, fever, arthralgia, and abdominal pain may also occur as results of splenic infarction[35]. The BC manifests as an acute leukemia with worsening of the symptoms present in the previous stages with bleeding, worsening fever, and secondary infections[5].

Atypical CML (aCML) affects about 5% of patients with CML, and includes absence of Ph chromosome and negative *BCR-ABL1* rearrangement, leukocytosis with left shift, splenomegaly, and marked myeloid dysplasia. These patients usually have a more unfavorable prognosis with poor response to treatment[39,40].

Considering that most patients are asymptomatic and the need for differential diagnosis with other hematological and systemic conditions, some tests are essential for the diagnosis of CML, such as blood count, bone marrow (BM) aspirate, cytogenetics, karyotyping, and qualitative and quantitative PCR[37]. The diagnostic workup, according to the latest European Leukemia Net (ELN) recommendations, should include physical examination with emphasis on the presence of hepatomegaly or splenomegaly, electrocardiogram, biochemical profile, complete blood cell count and differential count, BM aspirate for morphology, and cytogenetics performed by chromosome banding analysis of Giemsa-stained metaphases as well as molecular study, preferably through reverse transcriptase polymerase chain reaction (RT-PCR) to detect, typify, and quantify the *BCR-ABL1* transcripts. The molecular testing is becoming widely available, replacing the cytogenetic monitoring[41].

Blood count

Blood count during the CP shows leukocytosis with left-shifted myeloid maturation, revealing immature basophilic and eosinophilic myelocytes and metamyelocytes[2,36]. Also, the platelet count can vary, being high or low, and anemia can also occur[36]. The low phagocytic activity of granulocytes is typical of CML and differentiates the condition from other chronic myeloproliferative disorders[38].

To consider that a case of CML is in its AP, the blood count must present, according to the World Health Organization (WHO), at least one of the following criteria: 10%-19% of blasts, ≥ 20% of basophils, splenomegaly or an altered platelet count not attributable to treatment, persistence or increase of the white blood cell (WBC) count (> 10×10^9/L) during treatment, or even resistance-related parameters[42]. ELN defines such condition as the presence of 15%–29% of peripheral blood blasts or more promising peripheral blood blocks > 30% with blasts < 30%, or a ≥ 20% growth in basophils and platelet counts that is not attributed to treatment[38].

According to WHO criteria, BC is defined by extramedullary accumulation of blasts or a ≥ 20% proportion of blasts in peripheral blood or BM[42], or ≥ 30% of blasts, according to ELN criteria[38].

Molecular findings in aCML can include anemia, thrombocytopenia, and low incidence of basophilia[39]. The WHO criteria for aCML includes leukocytosis (WBC count ≥ 13×10^9/L) due to increase of neutrophils and their precursors with dysgranulopoiesis, neutrophil precursors ≥ 10% of leukocytes, minimal absolute basophilia (< 2% of leukocytes), no or minimal absolute monocytosis (usually < 10% of leukocytes), and blasts < 20% in the blood and BM[42]. The criteria also includes hypercellular BM with granulocytic proliferation and dysplasia, which can also occur in erythroid or megakaryocytic lineages, and the differential diagnosis with primary myelofibrosis, polycythemia vera, or essential thrombocythemia[42-44].

BM aspirate

BM aspirate is mandatory in all cases of suspected CML. The test can provide very important information, which allow for confirmation of the diagnosis and to determine the disease staging based on the percentages of blasts and basophils[5]. Besides, BM aspirate also allows analyses for the percentages of promyelocytes, myelocytes, and eosinophils[2]. According to the WHO criteria, the presence of 10%-19% of blasts in the BM is characteristic of CML in an AP[42]. The ELN criteria, on the other hand, characterizes an AP based on the presence of 15%-29% of blasts in BM or blasts plus promyelocytes > 30%, with blasts < 30%[43].

Cytogenetics

Regarding cytogenetics, an important finding is the hybrid gene *BCR-ABL1* and/or the t (9; 22) (q34.1; q11.21), which is pathognomonic for CML. The Ph chromosome is present in 95% of the CML patients and variant Ph chromosome translocations can involve three or more chromosomes[45]. Moreover, cytogenetic is useful to detect additional genetic abnormalities, being important to monitor the clonal evolution and CML progression. Secondary abnormalities usually include trisomy 8, isochromosome 17, and duplicate Ph chromosome, besides other abnormalities in small frequency, such as trisomy 19, trisomy 21, trisomy 17, and deletion 7[37,46].

Morris *et al*[47] described variant Ph translocations, involving *BCR, ABL1*, and additional chromosome sites, but the prognosis of *BCR-ABL1* variants is still controversial. The trial informs that patients with apparently normal karyotype can have the *BCR-ABL1* fusion gene only detected by more sensitive molecular techniques[47,48]. The presence of t (9; 22) (q34; q11.2) confirms the diagnosis, whereas additional abnormalities indicate CML progression. Possible variant Ph translocation

and complex BCR-ABL1 rearrangements require fluorescence in situ hybridization (FISH) analysis to confirm the clinical condition[47]. Cytogenetics is the gold standard for Ph chromosome detection, despite its low sensitivity (about 1%-5% of Ph-positive cells), the need for BM, and cell culture, which may result in delay of diagnosis[49]. This process is used in most medical centers and clinical trials, especially to detect additional chromosome abnormalities and clonal evolution. Unfortunately, karyotyping is still laborious, expensive and time consuming[50,51].

BM analysis by cytogenetics can also be used in the diagnosis of a BC, being an important predictor of the transformation of blasts. Besides, flow cytometry or cytochemistry can be used to define if the BC is of the lymphoid or myeloid type[52].

Cytogenetics in aCML can also show abnormalities, even though there is no specific molecular abnormality for that condition and multiple mutations are often present in various combinations[53]. The cytogenetics assists in the differential diagnosis with chronic neutrophilic leukemia and other myelodysplastic conditions, but clinical and laboratory findings, as mentioned above, are preponderant[54].

FISH

The FISH analysis relies on the colocalization of large genomic probes specific to *BCR-ABL1* genes and rapidly identifies specific genomic abnormalities, detecting *BCR-ABL1* rearrangements when cytogenetics is negative or when metaphase cells are not obtained[51,55]. The FISH technique is very useful in cases that lack cytogenetic evidence, revealing *BCR-ABL1* rearrangements and undetected t (9; 22) in an apparently normal karyotype, documenting Ph-negative rearrangements[55]. Among these genomic abnormalities, the prognosis and treatment seem to be similar, and the impact on survival is still unclear[56].

When combined with cytogenetics, the FISH can provide relevant information, such as deletions, *BCR-ABL1* fusion gene, and other translocations. Nevertheless, it is not able to monitor clonal evolution as cytogenetics does and its results depend on high quality commercial probes. The FISH is commonly used in medical centers and presents a low false-positive rate[49]. Nonetheless, FISH test is expensive and not widely available, requiring equipment and trained staff able to perform and interpret the test result[57,58].

Huntly *et al*[59], in their study, divided the FISH technique into two available types. Extra-signal BCR-ABL FISH probes flank the breakpoint of *BCR-ABL1* gene and cover 5´ABL genes. It promotes the detection of *BCR-ABL1* on the derivative chromosome 22 and reveals deletions on der(9) chromosome. Double-color Double Fusion *BCR-ABL1* FISH probes detect the fusion genes on chromosomes 9 and 22, revealing in 5′*ABL* and 3´*BCR* deletion on der(9)[59]. In this method, only larger deletions are found. FISH analysis is faster than using karyotype due to its independence of cell culture[51].

RT-PCR

RT-PCR technology is mainly used to determine the frequency of *BCR-ABL1* fusion transcript variants, detecting the M-bcr transcripts[49]. The RT-PCR is a technique that amplifies the region around the splice junction between *BCR* and *ABL*, detecting minimal residual disease[5], which is fundamental for CML diagnosis and monitoring. RT-PCR can be divided into qualitative, useful for diagnosis, and quantitative, ideal for monitoring residual disease[55]. Baccarani *et al*[60] showed that residual leukemia cells are only detected through RT-PCR[60], which emphasizes the importance of the method in these scenarios.

This technique is ideal for Ph-negative CML, detecting *BCR-ABL1* transcripts, and when the karyotype tests cannot be done. The RT-PCR does not face certain cytogenetic limitations, such as the dependence on BM metaphases and cell proliferation. Multiplex RT-PCR is also able to identify the molecular configuration profile. Furthermore, diagnosing Ph-negative patients with *BCR-ABL1* rearrangements is indispensable since the treatment options in that context are very similar to the ones available for Ph-positive CML. Multiplex RT-PCR uses peripheral blood, avoiding BM punctures, and it is faster than cytogenetics and conventional PCR, which favors the use of this technique[49,61]. However, RT-PCR standardization requires appropriate equipment, reagents, calibrators, and staff training, increasing the test-related costs. In Brazil, few laboratories are able to perform the RT-PCR test, and its costs are not covered by the public national health system[62].

Next-generation screening

Next-generation screening (NGS) is a promising technique expected to improve CML diagnosis. NGS is able to detect *BCR-ABL1* transcripts variants down to 1% abundance

and TKI-resistant mutations. *BCR-ABL1* kinase domain (KD) mutation is a mechanism of resistance in CML, especially to TKIs, observed in 50% of the patients in which imatinib treatment is unsuccessful. However, NGS faces obstacles for its implementation, such as high costs and prolonged runtime. The NGS testing can be useful in cases that do not respond to TKI treatment, allowing therapy optimization before CML transforms into BC[63,64].

In the Soverini *et al*[65] trial, 236 CML patients with failure and warning TKI response were analyzed by conventional cytogenetics (Sanger Screening, SS) and NGS. Fifty-one patients who were negative for mutations by SS showed low-level mutations on NGS. Furthermore, NGS identified additional low-mutations that were not detected by conventional cytogenetics in 29 out of 60 patients positive for mutations by SS. Hence, NGS was able to identify mutations that were undetectable by SS in 34% of the patients, being crucial for prognosis and clinical decisions[65]. With cost reduction, additional studies, and its implementation in clinical practice, NGS is expected to improve CML diagnosis and therapy, besides advances in drug development for patients with treatment failure[66].

Differential diagnosis

CML can be difficult to differentiate from other myeloproliferative or myelodysplastic syndromes. Polycythemia vera can manifest with leukocytosis and thrombocytosis. Individuals with primary osteomyelofibrosis may present with splenomegaly, neutrophilia, and thrombocytosis, for example[5,35].

Other conditions can be distinguished through specific laboratory findings (including negative Ph chromosome). Chronic myelomonocytic leukemia has dysplastic characteristics, cytopenia, and more intense monocytosis than CML as well as absence of basophilia[35,38].

CML with the P230 BCR-ABL transcript is associated with predominant neutrophilia, which can lead to a misdiagnosis of chronic neutrophilic leukemia. Therefore, cytogenetic evaluation is very important in all patients. Some CML patients may have isolated thrombocytosis without leukocytosis. This finding can be due to an essential thrombocytosis, but basophilia, which is usually present in CML, as well as cytogenetic and molecular tests may aid in the differential diagnosis[5,38].

Molecular monitoring

Cytogenetic analysis is important for diagnosis, prognosis, and monitoring of therapeutic response[67]. All patients should undergo a BM examination to establish the diagnosis, assess the percentage of blasts and basophils, and perform cytogenetic analysis to confirm the presence of the Ph chromosome and to exclude clonal evolution, particularly i (17) (q10) −7/del7q, and 3q26.2 rearrangements, which are associated with a relatively poor prognosis[5,35,55,68].

The presence of the Ph chromosome should be monitored by conventional cytogenetic analysis to obtain complete cytogenetic response (CCR) or molecular response (MR) by analysis of transcription levels of *BCR-ABL1* by RT-PCR and can correlate with prognosis[32,69,70]. The FISH is recommended for diagnosis in cases where the Ph chromosome is not detected by classical cytogenetics. FISH and RT-PCR are valuable tools in the identification of individuals with Ph-negative BCR-ABL-positive CML. In around 5%-10% of CML patients the Ph chromosome is not detectable by conventional cytogenetics and, among them, some may have submicroscopic *BCR-ABL1* aberrations, or more complex translocations in addition to the classic breakpoints of chromosomes 9 and 22[71].

The cytogenetics control can be used for frequent monitoring of a patient at risk of needing a change in therapy in case of treatment failure. Considering that FISH can quantify proliferating neoplastic cells in metaphase and non-proliferating cells in interphase, it used to be used for diagnosis and to analyze response to therapy using either peripheral blood or BM[72]. However, this technique has been replaced and it can only substitute chromosomal analysis in CML monitoring if BM cells are not obtained and/or for the definition of complete cytogenetic remission (CCyR)[69]. In addition, in patients with atypical transcripts, its use may be necessary for monitoring disease progression[41].

According to ELN, the monitoring of CML after the diagnosis is carried out with blood cell counts and differential cell counts as well as RT-PCR at least every 3 mo. A good sensitivity in RT-PCR is essential to adequately quantify the *BCR-ABL1* transcripts, guiding the therapeutic decision by achieving the milestones. The evolution of RT-PCR for CML diagnosis and monitoring has enhanced its cost-effectiveness and reduced invasive procedures[41].

Monitoring the cytogenetics and MR to treatment emerged as a success factor to

deal with a long-term disease. Patients with rapid cytogenetic or molecular remission (before 3 mo) and CCyR have a favorable prognosis[73]; more than 70% of them remain alive after 10 years. Karyotype is crucial in post-remission therapy decisions and molecular factors will determine treatment in individuals with normal karyotype[69].

CURRENT TREATMENT OF CML

The CML treatment has changed over time, as new medications are developed, especially those that are able to provide a balanced risk *vs* benefit ratio. Some of the theories that support the use of immunotherapy in the treatment of CML are related to the fact that this leukemia has a slow development as well because the leukemic cells are accessible since they are found in the blood and in the lymphatic system. Moreover, these cells have a very specific tumor antigen — the aforementioned BCR-ABL oncoprotein[74]. CML is considered one of the most sensitive neoplasms to immune manipulation, in addition to having well-established therapies that allow an important reduction in tumor mass. Given this, the last 20 years deserve to be highlighted for an important progress in the understanding of tumor immunology, whether in the area of passive or active immunotherapy. With regard to passive immunotherapy, hematopoietic stem cell transplantation (HSCT) and the donor leukocyte infusion can be mentioned, while vaccines are part of active immunotherapy[75].

TKIs

TKIs are the current standard treatment for patients with CML. After all, they have been able to effectively prolong patient survival, along with their rates of cytogenetic and molecular responses[74]. The main TKIs used are imatinib, desatinib, nilotinib, bosotinib, and ponatinib. Thus, some of their main aspects will be discussed here.

Imatinib is considered a first-generation TKI as it is a pioneer in the activity of inhibiting platelet-derived growth factor (PDGFR), KIT, and ABL, with successful clinical development, being approved for the treatment of CML in 2001. This is the gold-standard treatment, as it results in more expressive cytogenetic and molecular responses and has fewer adverse effects than interferon-alpha (IFN-a)[75]. The half-life of imatinib is approximately 14 h in the human body. Comparing healthy individuals with CML patients of equivalent ages, this drug revolutionized the treatment and prognosis of CML, promoting similar survival rates between these populations[76]. Recent reports suggest that almost half of CML patients treated with imatinib who obtained a lasting complete MR are able to discontinue treatment without relapse[77]. The number of natural killer (NK) cells appears to be an interesting parameter to be used as a prognostic factor for treatment with imatinib. After all, some studies have shown an increase in NK cell count along with the successful interruption of this immunotherapeutic[78,79]. In addition, some studies report that imatinib induces a complete hematological and cytogenetic response in approximately 83% of patients with CML for 10 years[80,81]. Some common adverse effects include edema (due to changes in the permeability of small vessels), weight gain, conjunctival irritation, bleeding from mucous membranes, diarrhea, and even skin rash. Less frequently, it can cause changes in liver enzymes, anemia, thrombocytopenia, and neutropenia[82,83]. Despite the increasing number of patients who obtain cytogenetic responses with this drug, about 30% of patients experience resistance to therapy, half of them showing the development of a point mutation in the ATP binding domain of the oncoprotein[84,85].

Dasatinib is a second-generation TKI and has become a central issue for research in the treatment of CML. So far, the number of CML patients who have benefited from treatment with this TKI remains unknown. Moreover, it has a shorter time on the market compared to imatinib[86]. Dasatinib is known as a "double inhibitor" because it inhibits, in addition to PDGFR and KIT, several members of the Scr family of tyrosine kinase. In addition, it has a half-life of 3-6 h and has a potency around 325 times greater than that of imatinib[87,88]. This medication is recommended for use when there is resistance to imatinib as well as other second- and third-generation TKIs[89,90]. The DASISION study demonstrated that the CCyR and Major MR (MMR) rates for the use of dasatinib at the dose of 100 mg, once daily, were higher and resulted in a faster and more effective response than that observed with the use of imatinib at a dose of 400 mg, once daily[91]. However, an important factor that can become an obstacle in the treatment of CML with this medication is the presence of pleural effusion as the main adverse effect in 30% to 40% of patients[92], which can lead to a discontinuation rate of 29%[93]. Other adverse reactions may include thrombocytopenia in higher rates when

compared with imatinib, which can lead to gastrointestinal bleeding[88]. Joint pain may be present and, rarely, pulmonary hypertension, which appears to be reversible with discontinuation of medication[94]. With regard to the follow-up of patients using this medication, the dose of dasatinib can be reduced to 50 mg per day, or even be administered every other day, if the disease is optimally controlled[92].

Nilotinib is a highly specific derivative and inhibits BCR-ABL with a potency approximately 25 times greater than that observed with imatinib, whether administered at a dose of 150 mg twice daily or 200 mg twice daily[95]. In addition, its use is approved for the treatment of patients with newly diagnosed CML in CP and patients with CML in CP or AP who are resistant or intolerant to prior therapies[96]. In the same review by Tian *et al*[96], they reported that this medication has a peak serum concentration about 3 h after administration, that its bioavailability is increased by about 82% when ingested with a high-fat meal compared to the fasting state, and that its metabolism is mainly *via* cytochrome P450 3A4. With regard to adverse effects related to this medication, studies have reported elevations in total bilirubin, acute pancreatitis, and, mainly, development of a kind of "metabolic syndrome"[97]. Valent *et al*[98] demonstrated in their study that, over a period of 5 years, nilotinib was associated with a prevalence of diabetes and cardiovascular events of 15% and 20%, respectively. These effects are of extreme concern, especially when this medication is used as a first line therapy, because of its potential lethality. However, another publication by Hochhaus *et al*[99] reported that even with these possible effects, the use of nilotinib 300 mg twice daily has a positive risk-benefit ratio. Therefore, continuous monitoring and evaluation of cardiovascular risk factors is required for patients selected for treatment.

Bosutinib is an oral SRC/ABL TKI licensed since 2012, which has shown to be effective in the treatment of resistant CML that does not harbor mutations in the T315l or V299L ABL KD, in all disease phases[100]. The main factor that distinguishes it from the inhibitory potential from other TKIs is that it does not block PDGFR or KIT[101]. Unlike previous TKIs, it has a relatively lower toxicity and its side effects are usually gastrointestinal disorders, such as diarrhea, which usually resolves spontaneously after 1 wk to 2 wk, cutaneous manifestations such as rash, in up to 20% of patients, and joint pain. In addition, bosutinib has been shown to have a safe cardiovascular profile, similar to imatinib, with a dose of 300 to 500 mg per day being recommended[102,103]. In addition, Tiribelli *et al*[104] provided data that demonstrate that bosutinib is a good therapeutic choice in patients who develop pleural effusion when using dasatinib[104].

With the use of TKIs, some patients may develop resistance due to the T315l mutation in the *BCR-ABL1* gene. Thus, third-generation TKIs like ponatinib have been developed. This medication is considered a pan-*BCR-ABL1* inhibitor that potently inhibits the T315I mutant and overcomes resistance based on mutations[105,106]. However, several studies have shown that ponatinib is associated with a high risk of developing hypertension, which can be severe, and even thromboembolic events. In fact, a first-line study comparing ponatinib, at a dose of 45 mg per day, with imatinib had to be suspended because of the high prevalence of cardiovascular events in the group using ponatinib[107,108]. Therefore, other studies using a reduced dosage of ponatinib, from 15 to 30 mg per day, have found a reduced incidence of cardiovascular effects. At the moment, the combination of ponatinib with antithrombotic medications is being tested[109]. Cortes *et al*[110] demonstrated in the PACE study that the estimated overall 5-year survival among patients using this medication was 73%, and evidenced the occurrence of treatment-associated rash (47%), abdominal pain (46%), and thrombocytopenia (46%)[110].

In view of the presence of the BC as a final phase in the evolution of CML and the high morbidity and mortality of patients affected by that condition, it is essential to establish an appropriate treatment for the crisis. The BC is the result of continuous BCR-ABL activity and oxidative stress[27,111,112] and its incidence has considerably decreased after the acquisition of TKI therapy. According to Saußele and Silver[28], studies have indicated an 8-year cumulative incidence of BC of 5.6% with the use of TKI compared to 12%-65% before the TKI era. Also in this work, there is a recommendation for treatment with a second- or third-generation TKI and use of chemotherapy, if necessary. The choice of chemotherapy should be based on the type of BC: If lymphoid, it can be used in an ALL-regimen with vincristine and prednisone, and in case of myeloid BC, an acute myeloid leukemia-regimen with anthracyclines and cytarabine is suggested. However, the BC response to TKI may be transient, which indicates that most cells are still sensitive to BCR-ABL inhibition. Thus, the most effective treatment for BC would be the early reduction of tumor burden and the elimination of BCR-ABL, which is still a challenge[113].

Resistance to TKIs

Although remarkable progress has been achieved in increasing the effectiveness of TKIs directed to BCR-ABL, resistance to these drugs is still being observed in CML patients. In such cases, stem cell transplantation is the only method that has proven to be effective in curing patients; however, this alternative is limited by donor availability[114]. Resistance to TKIs occurs in less than 10% of patients. On the other hand, persistence of residual quiescent cells affects the majority of patients, leading to chronic use of medication. Although only a part of patients become resistant to the treatment, there is a range of heterogeneous factors leading to persistence and resistance[115]. Patients who do not respond to drugs at the beginning of treatment have a primary resistance, whereas CMLs that initially respond to therapies and subsequently become resistant to them are classified as secondarily resistant[116]. Moreover, the resistance mechanisms are divided into two broad groups, namely BCR-ABL-dependent and BCR-ABL-independent mechanisms[117].

BCR-ABL-dependent resistance

Concerning the BCR-ABL-dependent drug resistance, mutations in the *BCR-ABL1* KD correspond to the most common type of resistance to TKIs. Such mutations promote steric modification of the protein structure and can lead to a stabilization of the active conformation of BCR-ABL, impairing drug ligation and, therefore, causing resistance. Among these mutations, stand out the ATP-binding P-loop between the amino acids 244 and 255, the C-loop between the amino acids 350 and 363, and the A-loop between the amino acids 381 and 402[118].

Among the countless mutation possibilities in the *BCR-ABL1* KD, the T315I is the most frequent when considering imatinib resistance, since it affects from 4% to 15% of CML patients who are resistant to that medicine[119]. It is characterized by the replacement of a threonine in the 315th position with an isoleucine, leading to a significant resistance to TKIs. Also known as "gatekeeper" T315I because it is a homologous mutation of the "gatekeeper" threonine residue, this mutation can cause a steric shock, impairing not only imatinib action, but also impeding nilotinib, dasatinib, and bosutinib to make up a hydrogen bonding with the protein. Therefore, this is an even more concerning mutation since it affects other generations of TKIs[120].

The sequential use of TKIs may contribute to the emergence of resistance-related mutations that can be classified as polyclonal, when two or more mutations separately affect the KD of distinct BCR-ABL proteins, or compound, when mutations simultaneously affect a single protein. It has to be emphasized that the main *BCR-ABL1* multiple mutations involve T315I and loop-P mutations[121].

Finally, some resistance mechanisms are associated with DNA repair pathways and genomic instability. In CML cells, BCR-ABL stimulates an excessive production of reactive oxygen species, which leads to genomic instability, predisposing the occurrence of potential mutations that can even take place in *BCR-ABL1* and cause TKI resistance[122,123]. In addition, the BCR-ABL kinase promotes the degradation of uracil DNA glycosylase, which is an important component of the DNA base excision repair pathway, an important mechanism for preventing the accumulation of mutations[124].

BCR-ABL-independent resistance

Among the several and heterogeneous BCR-ABL-independent resistance mechanisms, evidence suggests the existence of a BCR-ABL potential to activate an autocrine mechanism that confers a partial or complete growth factor autonomy. CD34+ cells of CML produce interleukin (IL)-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF), which promotes STAT5 phosphorylation. As a result, these proliferating cells are protected by GM-CSF against imatinib and nilotinib through the JAK2/STAT5 pathway. Furthermore, the CML resistance has been related to the overexpression of various other proteins, namely forkhead box protein O1, protein kinase C Eta (PRKCH), the SRC kinase family, 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3 (PFKFB3), B-catenin nuclear protein, akirin-2, tumor progression locus 2, nuclear factor-kappa B (NF-kB and NF-kB-p65), and MEK/ERK[118].

The BCR-ABL-independent mechanisms can even occur through membrane transporters. Since these components are essential for the availability and consequent effectiveness of the drug, the CML therapeutic process can be negatively influenced if they are affected[118]. The organic cation transporter-1 (OCT-1) works as a cell influx pump for imatinib. Therefore, adequate OCT-1 functioning is associated with better MMR rates, event-free survival, and global survival in CML patients in imatinib use[125]. On the other hand, the ineffectiveness of this transporter can lead to imatinib resistance. Regarding asciminib resistance, the efflux of this drug involves mainly the

ABCG2, which, if overactivated, can reduce the intracellular levels of this drug to undetectable levels[126].

If leukemic stem cells in CML are TKI-resistant, such resistance tends to persist in long-term therapy. Despite the limited knowledge about the mechanisms through which the leukemic stem cells become resistant to the current therapies, it is believed that it may be associated with the quiescence of these cells, which is controlled by intrinsic regulatory mechanisms and extrinsic signals from the microenvironment. Evidence has suggested that a stop in the cell cycle of such stem cells induced by specific signals from stromal cells may lead to resistance[127].

The frequency of chromosomal abnormalities in Ph chromosome-positive cells is positively associated with the severity of the disease. The most common chromosomal aberrations are an additional Ph chromosome, chromosome 8 trisomy, trisomy 19, and anomalies in the chromosome 17, and all of them are associated with imatinib resistance[116]. In addition, alterations in gene expression as a result of genetic modifications contribute to the resistance in CML. Among these alterations, stand out DNA methylation, histone modification, and noncoding RNAs[118].

New drug generations

Given the growing prevalence of TKI resistance in CML, various clinical trials have been conducted in order to make new drug options available for the patients affected by this disease. The following subtopics highlight the most promising alternatives in this context.

Asciminib

Asciminib is one of the most promising drugs among those aiming to be alternatives in the TKI resistance scenario. This medicine is an allosteric inhibitor that binds to the myristoylation site and alters the conformation of the KD to keep it inactive, and has the negative and muted *BCR-ABL1* as its target; that is, *ABL1* mutations such as gatekeeper T315I are not able to promote resistance to this drug[128]. A recently published phase 1 study observed an asciminib activity in 150 individuals, from which 141 manifested CML and 9 had accelerated CML with resistance or severe side effects with the use of at least two ATP-competitive TKIs. A complete hematological response was achieved in 92% of patients with a hematologic relapse, whereas 54% of those who did not initially present a CCR obtained a CCR. Interestingly, 28% of T315I carriers had a considerable MR during 12 mo of follow up[129]. In addition, the combinations between asciminib and other competitive TKIs may be useful in preventing the emergence of other mutations since the asciminib target site differs from the target sites of the other drugs. In that context, another study showed that the ponatinib-asciminib combination may be useful since asciminib is a substrate of the ABCG2 ATP-binding cassette transporter, whereas ponatinib is an ABCG2 inhibitor that can, partly, neutralize the positive regulation of ABCG2 as a resistance mechanism[130].

Considering asciminib as a promising drug to deal with the resistance and side effects of TKIs and aiming to increase the speed of response and the rates of deep molecular remission, there are currently phase 1[131] and phase 2[132,133] studies evaluating therapy with asciminib alone or combined with nilotinib, imatinib, and dasatinib. These studies aim to validate its safety, tolerability, and potential as first-line therapy. The preliminary results of the phase 1 study[131] published showed that asciminib achieved response in patients heavily pretreated, with unacceptable TKIs' side effects, failure in ponatinib treatment or T315I mutation, presenting as a promising alternative in these cases[129].

Radotinib

Radotinib is a BCR-ABL oral inhibitor indicated for newly diagnosed CML patients and for those with resistance to at least one TKI. Besides inhibiting the salvage-type BCR-ABL kinase, radotinib also inhibits other kinases, such as PDGFRα, PDGFRβ, c-kit, and SRC, when used in higher concentrations. However, this drug presents no consistent effectiveness against T215I[134]. A phase 3 study carried out in various Asian countries compared radotinib *vs* imatinib in newly-diagnosed CML. The results showed significantly higher and faster MMR rates in the radotinib group after 12 mo and 48 mo of follow-up[135].

Danusertib

Aurora kinases are serine/threonine kinases that are crucial for coordinated cell division. They participate in the maturation of centrosomes, in the formation of mitotic fuse, in chromosomal segregation, and in cytokinesis. In that context, danusertib

promotes the inhibition of the catalytic domain of aurora kinases as well as inhibits ABL and its mutated variations, including T315I[136]. This drug takes advantage of the crystal structure of the T315I KD and binds to the ATP-binding pocket of the enzyme active conformation, neutralizing the steric hindrance resultant from the substitution of threonine for isoleucine[137]. A phase 1 study about danusertib, that included 37 patients, showed subtle positive responses in patients with TKI-resistant CML in accelerated and blastic phases who carry T315I as well as in Ph chromosome-positive ALL. Among CML patients, 20% achieved a complete hematologic response and a CCR was observed in one individual[136].

HQP1351

HQP1351 is currently being clinically analyzed as a new generation multikinase inhibitor and it is expected to be useful in the treatment of TKI-resistant CML. Its main targets are the mutated wild-type BCR-ABL and KIT, including T315I. A phase 1 clinical trial administered HQP1351 in different doses in 101 patients, among which 87 had CP CML and 14 had AP CML. All individuals manifested resistance to other TKIs and 63% of them were T35I carriers. The results showed a complete hematologic response in 95% of individuals. Moreover, 69% of participants had a major cytogenetic response, 61% had a CCyR, and 37% had a MMR during a follow-up of 12 mo[138].

Immunotherapy

Vaccines are important immunotherapy components, being considered active immunotherapies. The advance in the knowledge on the BCR-ABL protein and its biological aspects has led to the development of a considerable diversity of studies based on the evaluation of vaccines based on peptides such as Pr-3, WT-1, and HSP70[139]. One of the major disadvantages of peptide-based vaccines is that they have the potential to induce an immune response to a few epitopes or just one, which increases the likelihood of an immune escape from CML cells[7,140]. On the other hand, studies on DNA vaccines have also been developed; however, their results are not yet available[141]. At the moment, therapeutic tumor cell vaccines modified by genes and halogenic tumor cell vaccines have also been tested. The former are advantageous because they carry a wide spectrum of antigens related to leukemia, promoting a wide and safe response. The latter have their cost-effectiveness as a positive point, and these cell lines seem to represent an unlimited source of immunizing antigens[31]. In addition, the appropriate time to use the vaccine is the period that follows normalization of the immune system activity with other therapeutic interventions against known immunosuppressive pathways. Thus, it is necessary to remember that immunotherapy should not be limited to the use of only one tool[142].

IFN-α was widely used in the treatment of CML in the 1980s, due to its disrupting role in the expression of the *BCR-ABL1* gene, in the activation of cell apoptosis factors, and in the recognition and elimination of CML-characteristic cells, in addition to promoting normal quiescent hematopoietic stem cell cycling[143-145]. The average rate of CCyR with IFN is 13%, ranging from 5% to 33%. This component targets highly quiescent leukemic stem cells, which have a high self-renewal capacity. Essers *et al*[145] demonstrated that INF-α was able to sensitize these stem cells. However, some adverse effects, such as flu symptoms, arthralgia, myalgia, neutropenia, and depression, contributed to the discontinuation of its use[145]. Currently, the discussion about the use of INF-α has returned, but in a combined use with TKIs. For example, Hjorth-Hansen *et al*[90] reported the results of their NordCML007 phase 2 study, combining dasatinib and pegylated IFN-α2b (PegIFN). A good tolerance of the combination with manageable toxicity and a marked increase in response rates after the introduction of PegIFN were observed.

Tumor cells have great efficiency in finding mechanisms that allow them to evade immunosurveillance[146]. As mentioned, one of these mechanisms occurs through PD-L1 present in CML cells, which, when attached to the PD-1 receptor in T cells, reduces their function[7,29]. Thus, some therapies seek to block the action of PD-1 and PD-L1 by anti-PD-1 and anti-PD-L1 antibodies, presenting, so far, promising results. Moreover, the adverse effects related to these drugs seem to not impair their use. Furthermore, a study that evaluated the use of anti PD-1/PD-L1 in 296 patients with solid tumors in advanced stages has found complete or partial treatment responses in these patients[147]. Some trials have shown that the use of immunomodulating antibodies are able to act by controlling the action of regulatory molecules in the immune system that compromise the functioning of T cells, being considered checkpoint inhibitors[148,149]. One of these inhibitors, nivolumab, has been undergoing phase 1 tests for use in CML[150]. A meta-analysis that included 6800 patients concluded that the use of drugs with anti-PD-1/PD-L1 action in cases of refractory or advanced cancers was highly

effective, further demonstrating that patients with hematological cancer had better results than those with solid cancer, in addition to performing allogeneic (allo) HSCT before using anti-PD-1/PD-L1 may be more effective in immune responses[151]. Moreover, studies performed in animal models demonstrated an increase in the survival rate of mice with CML, which were treated with αPD-L1 monoclonal antibody. This drug would block the PD-1/PD-L1 interaction and contribute positively to the regulation and action of immune system cells such as leukemia-specific cytotoxic T lymphocytes, CD8+ T lymphocytes, $CD4^+$ T cells, and, possibly, B lymphocytes, stimulating the immune response to the tumor[152]. Corroborating the previous research, the inhibitory pathway of the PD-1/PD-L1 interaction was able to stimulate an *in vitro* production of the IL-2, a Th1 profile cytokine, in an environment with CML cells, suggesting a possible positive effect on the activation and action of T lymphocytes[153].

Allogeneic transplantation

During the 1970s, Donnall Thomas' work showed the efficacy of the allo HSCT as curative therapy for CML, becoming the first-line treatment in patients in CP younger than 55-years-old[154,155]. With the emergence of TKIs, allo HSCT is no longer the first-line therapy, since 95% of those affected by CML are diagnosed in the CP, in which TKIs perform better[156-158]. The allo HSCT, as well as the TKIs used in the main therapy, has an excellent response against clonal cells, and the procedure-related morbidity and mortality has dropped sharply in recent years, with a consequent reduction in complications, such as graft versus host disease (commonly known as GVHD)[159]. The procedure is performed in patients of all ages, mainly in the sixth decade of life, but elderly patients less often meet the inclusion criteria for transplants. However, the age is not enough to determine the risks and benefits involved in the transplantation procedure[160-162].

With the improvement of treatment with TKIs and its effectiveness, only 6% of patients progress to AP or BC. However, in addition to the original mutation, other cytogenetic changes may occur, which limits the action of some first-line therapies[163]. As a rescue therapy, allo HSCT should be reserved for patients who are newly diagnosed in AP and BC phases or who are resistant to TKIs in these phases (including to ponatinib)[164]. This resistance may occur due to the T315I mutation in the coding domain of the *BCR-ABL1* gene[10]. Although this demonstrates a worse prognosis, it also raises the possibility of performing an allo HSCT, which has a healing potential[27].

In the first-line treatment, if there is resistance, it is acceptable to consider using the second-line treatment, and in case of subsequent resistance, the allo HSCT should be considered[165]. In general, regardless the line of treatment, if the molecular or cytogenetic response to two or more TKIs is suboptimal (that is, *BCR-ABL1* transcription > 1% or an in CCR with Ph^+ > 0%), an allo HSCT should be quickly considered, checking the compatibility of the human leukocyte antigen between patient and siblings, as well as searching for other possible donors in BM banks[41,51].

TKI therapy before and after transplant

Many patients undergo TKI therapy prior to allo HSCT[166]. Some studies demonstrate that it is related to a higher overall survival (measured from the transplant) if compared to patients who had not previously used TKI (in this case, imatinib) during first CP[167].

Another study (which analyzed 449 patients in AP, in second CP, and in BP of CML) found that the previous results do not apply to the advanced stage of the disease, so the use of these drugs before the allo HSCT did not alter the overall survival, nor leukemia-free survival, recurrence, treatment-related mortality or GVHD when compared to the group that had not previously used a TKI[168]. However, the indicators already known for performing allo HSCT should still be the main factors in determining prognosis[169].

After performing allo HSCT, BCR-ABL may still be detected for a few months, considering that allograft adaptation is not immediate[170]. However, checking for the presence of BCR-ABL (which can be negative, fluctuating or positive) during the next 6 mo can predict a prognosis for cure or relapse[171]. In this sense, the administration of a standard dose of imatinib as a prophylactic therapy against possible relapses after allo HSCT can be performed, since it can potentiate anti-tumor effects by dendritic cells[172]. Compared to the previous standard, this practice is more effective if performed in CP of CML, being less useful if performed in advanced phases[170-174].

Indications and risks of performing the allo HSCT

In the era of TKIs, the indication for HSCT varies according to the stage at diagnosis and should never be dissociated from the risks related to the disease[175,176]. In this way, the European Blood and Marrow Transplant Group (EBMT) developed a risk and chance score for performing the allo HSCT based on five criteria, including the stage of the disease, the age of the recipient, the type of donor, the donor sex, and the interval between diagnosis and transplantation. The sum of the scores varies from 0 to 7 in each item (Table 1)[177,178].

At CP, allo HSCT should be considered based on resistance to second-generation TKIs, being it first- or second-line, as well as on the ponatinib failure after 3 mo of treatment[89]. CP patients who fail in the second line of treatment with TKIs, regardless the EBMT risk score, should be immediately referred for an allo HSCT and the ABL mutational analysis must be performed to better choose the therapy before transplantation[179].

The CML advanced phase is present in the risk analysis carried out by EBMT, ranging from 1-2 points and, therefore, having an impact on the transplant result[180]. For cases in which there is progression or an initial diagnosis in AP, the chance of TKI failures is enormous, and allo HSCT should be done as soon as the condition stabilizes[181]. Patients in AP CML tend to benefit better from allo HSCT compared to treatment with imatinib, under the influence of two criteria: The diagnosis was over 12 mo ago or the amount of blasts in the peripheral blood is greater than 5%[182]. For those patients in BC with a frank explosion, allo HSCT is not recommended, and healing through transplantation occurs in a small number of cases in this phase of the disease (< 10%)[41,183]. Thus, the decision to perform a transplant must consider numerous factors such as the risks of the procedure and the disease stage/progression. A study that analyzed a Swedish database showed a greater post-transplant overall survival at 5 years for individuals in the first CP (96.2%) and lower for those in AP or BP (36.9%)[184].

NEW PERSPECTIVES IN THE CML TREATMENT

Faced with the possibility of therapy-resistant cells to conventional drugs[185] or other treatment problems, whether by non-remission or progression of the disease and possible adverse effects, new research is looking for alternatives that treat the CML more effectively and improve the life quality of the patients[186]. The following topics gather some of the most relevant therapeutic strategies in this context.

Peroxisome proliferator-activated receptor gamma inhibitors

Peroxisome proliferator-activated receptor-gamma (PPAR-γ) is a transcription factor that may interfere with the action of the protein OCT-1, which, as previously explained, is related to the transport of imatinib into *BCR-ABL1*+ cells, among other functions[187]. The ligands of PPAR-γ, such as pioglitazone, are drugs currently used to treat diabetes that have demonstrated utility in the therapy of CML[188]. Studies have reported the use of these medicines as therapies for some neoplasms, acting as an inhibitor of tumor cells growth or increasing apoptotic capacity, decreasing cell proliferation[189].

Once this therapeutic class is connected to its receptor, a negative interference in the transcription of the RNA STAT5, an oncogenic mediator related to the stability of the CML cells, occurs and an increase in the OCT-1 expression is observed, making the cell more sensitive to the imatinib action[190]. Moreover, it was reported that pioglitazone combined with imatinib increases the apoptotic capacity of tumor cells by a positive regulation of caspase 3, which may mean that a combined therapy with these two drugs can be an alternative for resistant CML treatment[186]. Telmisartan, a partial agonist of the PPAR-γ, has shown to be effective in circumventing the resistance to imatinib, possibly being more effective than pioglitazone[185].

There are reports of patients who had improvements in clinical conditions with the combined use of TKIs and PPAR-γ inhibitors, getting a more favorable MR than the patients who used only imatinib. However, more studies that assessed the effectiveness of this combination in the treatment of CML still are necessary[188].

Immune modulation

WT1 peptide vaccine: The *WT1* gene was initially isolated from the Wilms' tumor, being responsible for its suppression. However, another form of this molecule, the wild-type WT1, showed to be highly expressed in hematopoietic neoplasms, such as

Table 1 European Group for Blood and Marrow Transplant risk score for allo hematopoietic stem cell transplantation

	Score		
	0 points	1 point	2 points
Age	Younger than 20 yr	Between 20 yr and 40 yr	Older than 40 yr
Disease stage	Chronic	Accelerated phase	Explosion crisis
HLA matching, donor	Brother with identical HLA	An unrelated donor	-
Donor-recipient gender	All other combinations	Female donor to male recipient	-

HLA: Human leukocyte antigen.

CML[191]. Therefore, due to its high levels and role in the oncogenesis process, this gene may become a possible therapeutic target, contributing to a better patient prognosis[192].

The WT1 peptide vaccine was able to stimulate the emergence of CD8+ cytotoxic T cells with combat specificity to the leukemic cells, suggesting a positive immunomodulation and specific antigen response[193]. Corroborating that, a phase 2 study also reported a favorable MR of the peptide vaccine use, which interfered in the *BCR-ABL1* gene transcription, evidencing a possible ability to assist in the CML treatment, and has also shown to be safe[194]. In addition, the combined use of the peptide vaccine and the imatinib made possible the BCR-ABL mRNA reduction, a marker of residual disease in the peripheral blood. Furthermore, it contributed to decreasing the imatinib doses necessary for the treatment, reducing the chances of side effects without interfering with the therapy effectiveness[195].

Peptides derived from the BCR-ABL gene

The peptides generated from the fusion gene *BCR-ABL* have been studied as possible therapeutic targets, because the use of oncogenic antigens in the treatment of some neoplasms has shown less ability to generate side effects once they act directly in abnormal cells, besides presenting a cure potential[196,197].

The use of the peptide vaccines proved to be secure and able to stimulate a favorable antigen-specific immune response, also improving CD4+ T cell responses, indicating possible efficacy in the CML treatment[198]. Moreover, a significant decline of BCR-ABL mRNA level in peripheral blood was detected after vaccine use[199]. Lastly, the combined therapy among the vaccine and imatinib may also reduce the doses of the TKIs necessary for the treatment, which may reduce the chances of possible side effects[141].

Dipeptidyl peptidase IV inhibitors

Dipeptidyl peptidase IV (DPPIV) is a membrane protein that, in leukemia cells, is able to degrade and interfere in the stroma-derived factor 1 action, a chemokine that is expressed in normal myeloid stem cells related to the interaction and stability of the hematopoietic niche[200]. DPPIV proved to be efficient in the differentiation of normal cells from leukemia cells, which may contribute to either diagnosis or prognosis of this disease, besides being a possible therapeutic target[201].

Patients who used gliptins, DPPIV inhibitors used to treat type 2 diabetes mellitus, along with CML treatment significantly reduced the BCR-ABL mRNA levels, indicating possible synergistic effects between the medicines. Furthermore, the gliptins were also able to inhibit the expansion and proliferation of CML cells, which may contribute to neoplasm control[200]. However, due to the scarce amount of research in this area, further studies need to be done aiming to evaluate the appropriate therapeutic doses[201].

Concluding, the new therapeutic approaches in the CML treatment emerge as an alternative to circumvent possible problems of conventional therapy. Although there are few studies in this area, the combined use of the TKIs with these new therapeutic options has already been related to improvements in the patients' clinical conditions and prognosis[202].

CONCLUSION

Although the knowledge about CML cytogenetics has been well elucidated, the role of innate and adaptive immunity in the prevention as well as in the development of this disease and the role of additional mutations should be understood to a broader extent, allowing the advancement of new therapies, aiming to improve the quality of life and survival of these patients. New drugs and the advancement of immunotherapy are essential to tackling TKI resistance and maintaining low residual levels of cancer cells.

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ORIGINAL ARTICLE

Basic Study

Autosegmentation of cardiac substructures in respiratory-gated, non-contrasted computed tomography images

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Abstract

BACKGROUND

Radiation dose to specific cardiac substructures can have a significant on treatment related morbidity and mortality, yet definition of these structures is labor intensive and not standard. Autosegmentation software may potentially address these issues, however it is unclear whether this approach can be broadly applied across different treatment planning conditions. We investigated the feasibility of autosegmentation of the cardiac substructures in four-dimensional (4D) computed tomography (CT), respiratory-gated, non-contrasted imaging.

AIM

To determine whether autosegmentation can be successfully employed on 4DCT respiratory-gated, non-contrasted imaging.

METHODS

We included patients who underwent stereotactic body radiation therapy for inoperable, early-stage non-small cell lung cancer from 2007 to 2019. All patients were simulated *via* 4DCT imaging with respiratory gating without intravenous contrast. Generated structure quality was evaluated by degree of required manual edits and volume discrepancy between the autocontoured structures and its edited sister structure.

RESULTS

Initial 17-structure cardiac atlas was generated with 20 patients followed by three successive iterations of 10 patients using MIM software. The great vessels and heart chambers were reliably autosegmented with most edits considered minor. In contrast, coronary arteries either failed to be autosegmented or the generated structures required major alterations necessitating deletion and manual definition.

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Similarly, the generated mitral and tricuspid valves were poor whereas the aortic and pulmonary valves required at least minor and moderate changes respectively. For the majority of subsites, the additional samples did not appear to substantially impact the quality of generated structures. Volumetric analysis between autosegmented and its manually edited sister structure yielded comparable findings to the physician-based assessment of structure quality.

CONCLUSION

The use of MIM software with 30-sample subject library was found to be useful in delineating many of the heart substructures with acceptable clinical accuracy on respiratory-gated 4DCT imaging. Small volume structures, such as the coronary arteries were poorly autosegmented and require manual definition.

Key Words: Autosegmentation; Autocontouring; Lung cancer; Radiation therapy; Heart substructures; Stereotactic body radiation therapy

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Core Tip: Autosegmentation is an attractive tool to reduce the labor involved with manual delineation of anatomy. However, it is unclear whether this approach is viable for all treatment conditions. Stereotactic body radiation therapy frequently utilizes respiratory gated, non-contrasted computed tomography imaging for radiation planning and involuntary heart motion as well as lack of intravenous contrast may impact the quality of generated structures. In our study, MIM software successfully contoured the great vessels and heart chambers yet failed in generating coronary arteries. We provide evidence that MIM software can reliably autocontour the larger cardiac substructures, but not coronary arteries or heart valves.

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INTRODUCTION

Cardiotoxicity is a significant concern in modern radiotherapy (RT). RTOG 0617 was a large, phase III trial investigating the utility of dose escalation in locally advanced non-small cell lung cancer (NSCLC), where the experimental dose escalated arm reduced patient survival, in part due to excessive cardiotoxicity[1]. In a follow up study, Thor *et al*[2] revealed that dose to specific cardiac substructures such as the atria, ventricles, and pericardium were highly correlated with overall survival. These findings have been supported by several other groups, who also demonstrate a dose-dependent relationship between cardiac substructures and outcome[3,4]. Despite these findings, definition of such structures is not standard in radiation planning at this time.

Conventionally, the heart is contoured as a single structure which encompasses the atria, ventricles, valve, pericardium, and coronary arteries. Individual definition and subsequent dose calculation to the cardiac substructures is not routinely performed. Delineation of cardiac subsites can be challenging due to heart and respiratory motion, lack of intravenous contrast on treatment planning computed tomography (CT) imaging, and anatomic variances[5-10]. Furthermore, contouring these structures can be time consuming and not necessarily practical in typical care[10].

Researchers have attempted to overcome these obstacles utilizing a number of approaches. For example, several groups have provided cardiac atlases for these subsites, including the recently closed clinical trial RTOG 1106 (NCT01507428) to provide guidance on how to define these structures[5,10,11]. Additionally, there is evidence that autosegmentation is a feasible option to mitigate the added labor associated with including these structures[6-10,12]. While this is a provocative strategy, this method may not be viable in all treatment conditions. For example, four-

dimensional (4D) CT imaging with respiratory gating is a treatment delivery technique for stereotactic body radiation therapy (SBRT) utilized by approximately 31%-54% of providers in the United States[13,14]. As this requires multiple scans to construct each phase of respiration, timed intravenous contrast is not possible and cardiac motion may be amplified. Therefore, it is unclear how well autosegmentation would perform in this setting. We investigated whether autosegmentation for cardiac substructures could be accurately employed on non-contrasted, respiratory gated imaging.

MATERIALS AND METHODS

Patients and CT simulation

We included patients who underwent SBRT for inoperable, early-stage NSCLC from 2007 to 2019. Data was collected under approval from the institutional review board at Roswell Park Comprehensive Cancer Center. CT simulation was previously described[15]. All patients were simulated in the supine position with a Body Fix immobilizer (Elekta, Stockholm, Sweden) with respiratory gating. Real-Time Position Management by Varian Medical System (Palo Alto, CA, United States) was utilized to assess respiratory motion. After evaluating tumor motion, treatment planning was performed on CT average of combined respiratory phases where tumor motion was ≤ 5 mm. With the exception of one patient who was treated using a breath-hold technique due to an irregular breathing pattern, all patients were treated using a respiratory phase-based 4D approach.

Cardiac atlas development and validation

Cardiac atlas development was approached in two phases. First, 20 patients who were planned for lung SBRT using 4DCT imaging were chosen for initial atlas construction. Cardiac substructures were manually contoured by a single senior Radiation Oncology resident in the Eclipse treatment planning system (Varian Medical System, Palo Alto, CA, United States) on a CT average scan. The 17 cardiac substructures were the four heart chambers (left/right atria, left/right ventricles), four coronary arteries (left main common, left circumflex, left anterior descending, right coronary artery), four heart valves (aortic, pulmonary, mitral, tricuspid), pulmonary artery, ascending aorta, descending aorta, whole heart, and superior vena cava. These structures were well defined by a previously described cardiac atlas and an example is provided in Supplementary Figure 1[5].

Secondly, these 20 patients were subsequently exported from Eclipse to MIM software (version 6.9.6, Beachwood, OH, United States) and a multi-patient atlas library pretraining to heart was generated for our lung SBRT patients. Using this atlas, 10 additional patients underwent autosegmentation in MIM. These generated structures were then reviewed and refined by the same Radiation Oncology resident, where the initial MIM's generated structure were saved, copied, and edited to improved accuracy. These edited structures were then added back to the MIM multi-patient atlas library. This process was repeated two additional times adding additional 10 patients every time to the library. These steps were taken to study if incrementally adding patients to the library of patients improves auto contouring accuracy as the MIM software does not recommend minimum number of subjects in the atlas. In two instances, patients were replaced after substantial problems were noticed in the autosegmentation caused by anatomic abnormalities due to a prior pneumonectomy in one patient and a significant hiatal hernia in another. The final number was 10 patients for each iteration in MIM. The entire process is detailed in Figure 1.

Structure evaluation

Autosegmentated structures by MIM were evaluated by two separate methods. First, autogenerated contours were evaluated manually by the Radiation Oncology resident. A score was given for each structure: 0 = No changes made; 1 = Minor changes made; 2 = Moderate changes required by structure was salvageable; 3 = Structure required deletion and replacement; 4 = Structure failed autosegmentation. Minor changes would describe cleaning up a border of an otherwise accurate structure, where in moderate changes the general shape of the contour may be accurate, but a larger number of edits were necessary. Second, the volumes of autocontoured structures and its edited sister structure were compared. In the setting where a structure failed autosegmentation, the volume measurement was left blank. Data were presented using GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, CA, United States).

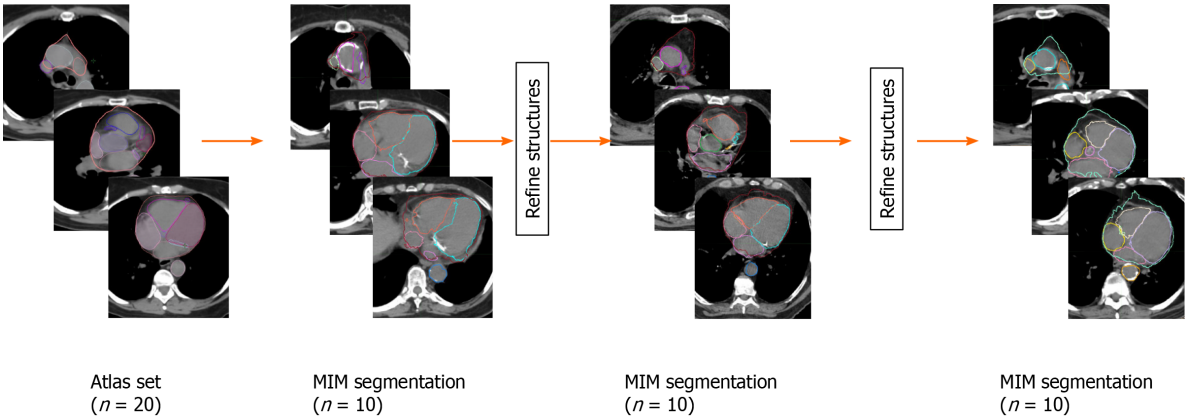


Figure 1 Project workflow of initial multi-atlas library construction, MIM autosegmentation, and subsequent edits of generated structures to add to the MIM atlas library.

RESULTS

Utilizing MIM software, cardiac substructures were autosegmented in 10 patients and these structures were then manually refined (Figure 1). This process then repeated two subsequent times and representative autosegmented structures following each iteration can be found in Supplementary Figures 2-4. The great vessels including the whole heart were reliably autosegmented with the majority requiring minor changes without a substantial improvement from additional samples (Figure 2). In contrast, coronary arteries either failed to be autosegmented or the generated structures required major alterations necessitating deletion and manual definition. Similarly, the generated mitral and tricuspid valves were poor whereas the aortic and pulmonary valves required at least minor and moderate changes respectively (Figure 2). The heart chambers, particularly the atria, were autocontoured well (Figure 2). As compared to structures generated with 20 samples, there was a general improvement in structure quality after 30 samples, however, this trend did not continue in the 40-sample group.

Additionally, volumes of the manually contoured and autosegmented cardiac substructures for the respective groups were compared. In general, MIM undercontoured structures when compared to the manually derived contours (Figure 3). This discrepancy was typically smaller for cardiac substructures with larger volumes such as the great vessels and heart chambers, whereas the difference was more profound in the smaller structures (Figure 3). There was excellent agreement in the volume of autogenerated and manual structures for the whole heart (Supplementary Figure 5). In some structures (right ventricle, descending aorta, pulmonary artery), more samples improved the volume discrepancy between manual and autocontoured structures (Figure 3). Unfortunately, often the autosegmented structures for the coronary arteries, mitral, and tricuspid valves were very small (< 0.1 cm^3) and essentially unusable.

DISCUSSION

In current study, we explored the feasibility of MIM autocontouring for cardiac substructures on respiratory gated, non-contrasted 4DCT scans. Following autosegmentation, the great vessels and heart chambers required minor to no changes on average, whereas coronary arteries, tricuspid and mitral valves often failed autosegmentation or required complete recontouring. The aortic and pulmonary valves required a greater degree of modification but ultimately were usable structures. Similar observations were seen on volumetric analysis between the autosegmented structures and their manually edited correlates.

Several atlases for cardiac substructures have been described. The current study defined heart subsites per Feng *et al*[5], who used contrast-enhanced CT images of breast cancer patients to provide a well-defined atlas which is similar to previous studies, including RTOG 1106 (NCT01507428)[5,11,16].

Prior reports have evaluated methods for autocontouring cardiac substructures. Kirisli *et al*[17] utilized a multi-atlas approach to autosegment the whole heart and

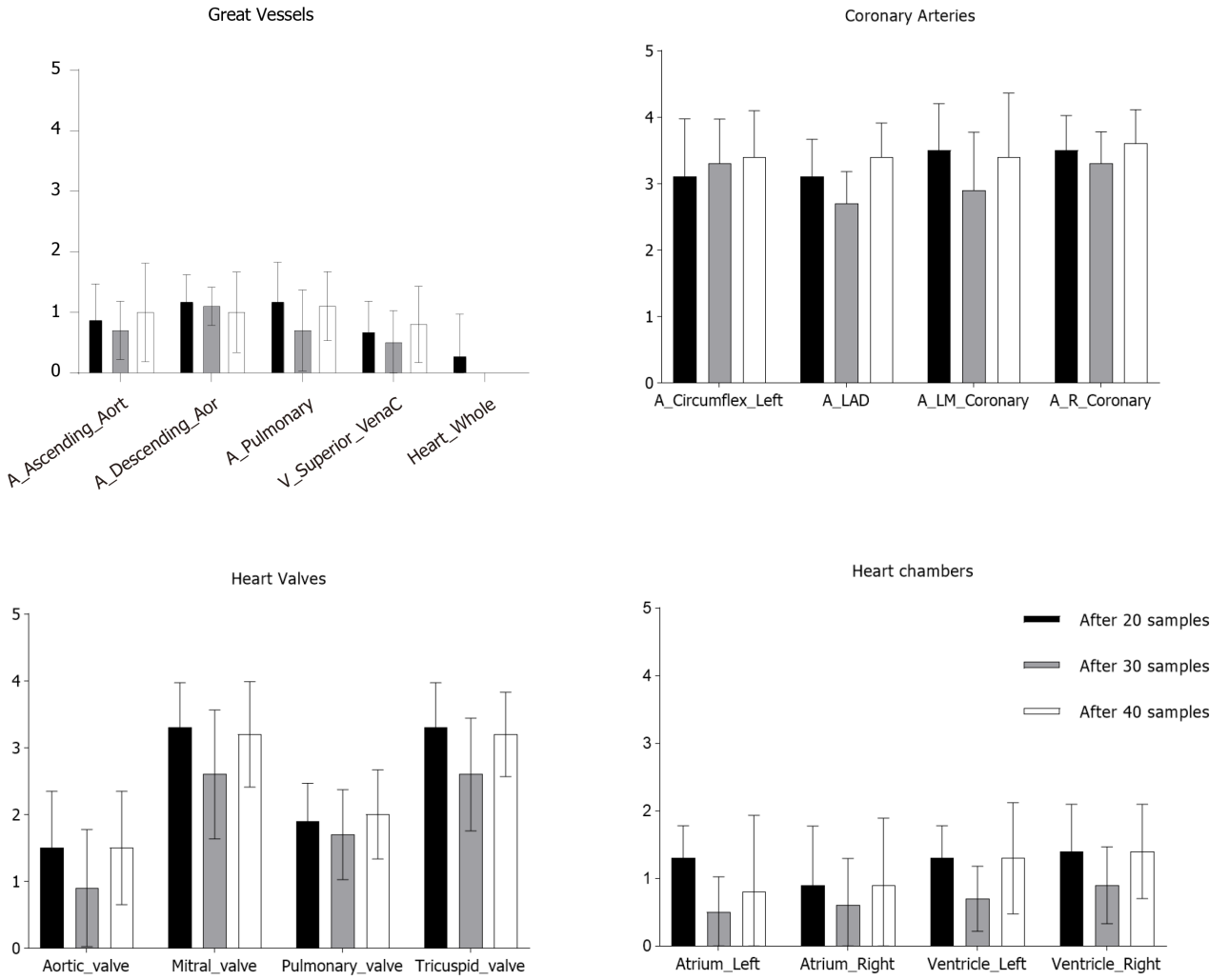


Figure 2 Physician-based evaluation of generated structures using atlas libraries comprised of 20-, 30-, and 40-patient samples (mean, standard deviation). Scale: 0 = No changes made; 1 = Minor changes made, 2 = Moderate changes required by structure was salvageable, 3 = Structure required deletion and replacement, 4 = Structure failed autosegmentation.

cardiac chambers using CT angiography. Shahzad *et al*[9] relied on contrast enhanced imaging for atlas generation, yet was then able to successfully autosegment structures on non-contrasted imaging *via* this template.

Others have demonstrated the reliability of non-contrasted images for atlas construction. In a breast cancer population, Finnegan *et al*[6] used multi-atlas segmentation in non-contrasted, free-breath CT imaging reporting similar success with the great vessels, whole heart, and heart chambers. Moreover, Luo *et al*[8] utilized non-contrasted, 4DCT images for multi-atlas autosegmentation finding minimal changes needed in generated structures with no dosimetric difference between autosegmented and manually derived structures. Additional groups have successfully used 4DCT imaging in deriving cardiac atlases and subsequent autosegmentation[3,10]. Consistent with our findings, these studies have reported success in autocontouring the great vessels and heart chambers, whereas the coronary arteries and heart valves have too much variability to be reliably used, despite a wide variety of planning techniques[3,6,8,10].

In the current report, the quality and volume agreement of the studied structures improved when using a 30 *vs* 20 patient atlas, however this trend did not continue when 40 patients were used. The reason for this discrepancy is likely due to anatomic variances. The study cohort consisted of patients planned for intrathoracic SBRT, a group that often has a high proportion of cardiac disease (manuscript in press). As such, it is not uncommon to see cardiac hypertrophy, atherosclerosis, implantable hardware (pacemakers, cardiac stents), and other cardiovascular pathologies within this patient population. These factors can ultimately impact the ability to accurately autosegment heart substructures either due to the relative high hounsfield units of

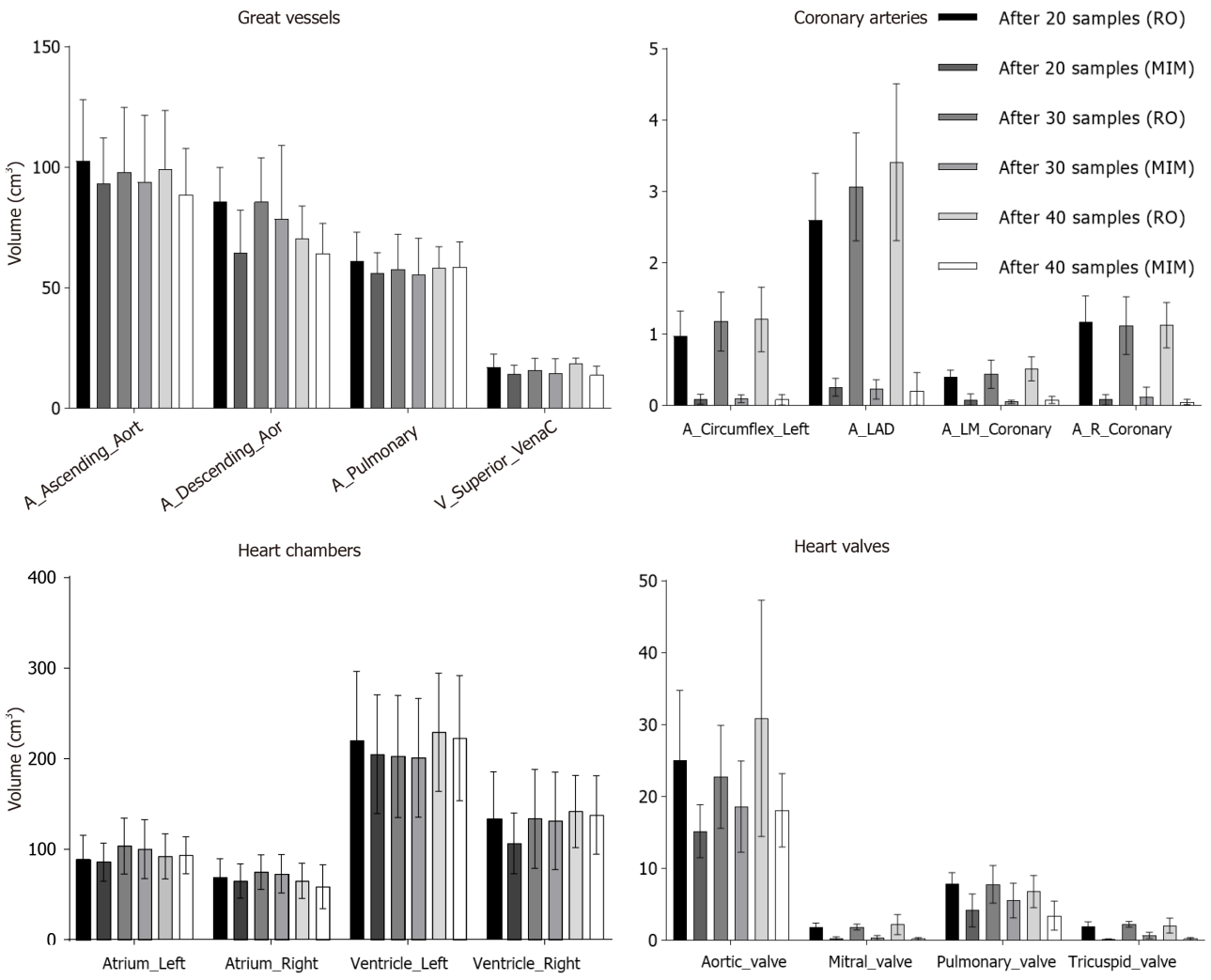


Figure 3 Analysis comparing the volumes of autosegmented structures (MIM) *vs* subsequent physician-edited sister (RO) (mean ± SD).

implants/calcium or differences compared to normal anatomy. Indeed, four patients within the 40-sample group who required a high degree of modification of autocontoured structures had such abnormalities (pacemaker and cardiac stents, 1 patient; sternotomy wires and atrial/ventricular hypertrophy, 1 patient; significant atherosclerosis and atrial/ventricular hypertrophy, 2 patients). Therefore, while increasing the sample number in the atlas database can improve the quality of generated structures, individual patient factors can still significantly impact autosegmentation.

There are several limitations in our study. The lack of intravenous contrast can limit the identification of cardiac substructures, particularly the coronary arteries and heart valves. However, given that these are small volume structures significantly impacted by cardiac motion and the difficulty that previous studies had with autosegmenting these subsites, contrast alone may not be sufficient to overcome this issue. Additionally, the conducting pathways of the heart cannot be reliably identified on CT[5]. Future studies incorporating cardiac magnetic resonance imaging could be useful in this setting. Lastly, while increasing the number of patients in the MIM atlas did not consistently improve contour quality in this study, given the limited number patients it is unclear whether this is applicable to much larger datasets. Strengths of this study includes the use of respiratory gated planning CTs, as this technique is commonly used for motion management in intrathoracic SBRT. While respiratory gating can amplify cardiac motion, the current study demonstrates that MIM autosegmentation is viable for the great vessels and heart chambers thus enabling a practical approach to dose calculation to these structures. Future studies will use this approach to evaluate radiation dose to cardiac substructures during SBRT.

CONCLUSION

Accurate segmentation of various organs in the heart is necessary to understand and correlate cardiac toxicities in the management of NSCLC lung cancer patients being treated using SBRT dose delivery technique. Even though human heart is associated with non-voluntary motion, thereby, exhibiting motion artifacts during imaging, use of MIM software with 30-sample subject library was found to be useful in delineating majority of the heart substructures with acceptable clinical accuracy except some structures having very little volume. Moreover, the autosegmented structures need to be evaluated on case by case basis by the treating physician as presence of calcifications or other sources of imaging artifacts like pacemaker, *etc.*, may inhibit the model to accurately auto segment the heart sub-structures.

ARTICLE HIGHLIGHTS

Research background

Cardiotoxicity from thoracic radiotherapy can significantly contribute to treatment related morbidity and mortality. Despite this, cardiac substructures are not routinely delineated in thoracic radiation planning.

Research motivation

Autosegmentation of cardiac substructures would allow for relative dose calculation to these subsites without the added labor of manual definition.

Research objectives

To determine whether autosegmentation software can be successfully employed for the cardiac substructures in patients planned using respiratory gated, non-contrasted computed tomography (CT) imaging.

Research methods

This retrospective study included patients who underwent stereotactic body radiation therapy (SBRT) for inoperable, early-stage non-small cell lung cancer from 2007 to 2019. All patients were simulated *via* CT imaging with respiratory gating without intravenous contrast. A 20-patient atlas of the cardiac substructures was manually constructed and used to facilitate autosegmentation *via* MIM software. A total of three iterations of autosegmentations were completed, each using 10 patients. Generated structure quality was evaluated by degree of required manual edits and volume discrepancy between the autocontoured structures and its edited sister structure.

Research results

The great vessels and heart chambers were reliably autosegmented with most edits considered minor. In contrast, coronary arteries either failed to be autosegmented or the generated structures required major alterations necessitating deletion and manual definition. Similarly, the generated mitral and tricuspid valves were poor whereas the aortic and pulmonary valves required at least minor and moderate changes respectively. For the majority of subsites, the additional samples did not appear to substantially impact the quality of generated structures. Volumetric analysis between autosegmented and its manually edited sister structure yielded comparable findings to the physician-based assessment of structure quality.

Research conclusions

Our study indicated that the great vessels and heart chambers can be reliable autocontoured using MIM software. On the other hand, autosegmentation for valves is inconsistent and poor for coronary arteries. Anatomic variances and/or implanted hardware may impact the quality of autosegmentation.

Research perspectives

Radiation heart dose is an important dosimetric parameter however dose tolerances for the cardiac substructures in conventional therapy and SBRT are not well-established. Therefore, artificial intelligence based contouring programs allow dose to be calculated to the select cardiac subsites without the added labor of manual definition.

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ORIGINAL ARTICLE

Retrospective Study

Racial disparities in immune-related adverse events of immune checkpoint inhibitors and association with survival based on clinical and biochemical responses

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Abstract

BACKGROUND

Immune checkpoint inhibitors (ICPi) cause various immune-related adverse events (irAE) with thyroid dysfunction as a commonly reported abnormality. There is increasing evidence showing positive association with development of irAE and survival. However, prior trials with ICPi had underrepresentation of minorities with < 5% African Americans.

AIM

To evaluate the association between development of irAE and survival outcomes among a racially diverse patient population.

METHODS

Data on patients with stage IV solid malignancies treated with programmed cell death-protein 1/programmed death ligand 1 blockers between January 2013 and December 2018 across MedStar Georgetown Cancer Institute facilities were

informed consent to the study because the analysis used anonymous clinical data.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: No additional data are available.

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retrospectively reviewed. Patients treated with cytotoxic T-lymphocyte-associated protein 4 inhibitors were excluded. Progression free survival (PFS) and overall survival (OS) were primary endpoints and were calculated using Kaplan-Meier methods and Wilcoxon rank sum test for comparison.

RESULTS

Out of 293 patients who met eligibility criteria, 91 pts (31%) had any grade irAE; most common AE were endocrine (40.7%) specifically TSH elevation, dermatological (23.1%) and rheumatologic (18.7%). Proportion of irAE was significantly higher in Caucasians *vs* African Americans (60.4% *vs* 30.8%), in patients with low programmed death ligand 1, lower LDH, older age, and those who had more treatment cycles with ICPi. Rate of progression was lower in patients with irAE (30.8% *vs* 46.0%, $P = 0.0140$). Median PFS (5.8 *vs* 3.0 mo, $P = 0.0204$) and OS (17.1 *vs* 7.2 mo, $P < 0.0001$) were higher with irAE. Statistically significant difference in OS (17.1 *vs* 8.6 mo, $P = 0.0002$) but not in PFS (5.8 *vs* 3.3 mo, $P = 0.0545$) was noted with endocrine irAE. No differences in survival were observed among other commonly reported irAE. Differences in survival among subgroups of patients with irAE are described.

CONCLUSION

Development of irAE positively correlated with improved PFS and OS as reported in previous studies. To our knowledge, this is the first study observing differences in OS favoring endocrine AE and Caucasian race. These factors may be potential surrogate markers of prognosis pending replication of these results in large-scale studies.

Key Words: Immunotherapy; Adverse events; Endocrine; Survival; Race; Minority

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Core Tip: Immune checkpoint inhibitors have revolutionized the treatment of many types of cancer, yet they cause various immune-related adverse events (irAEs), notably thyroid dysfunction. Development of irAEs has been reported as a surrogate marker of improved survival. However, minority racial group representation is a limitation in immunotherapy trials. In this retrospective study, we evaluate the association of development of irAEs to survival outcomes in patients treated with immune checkpoint inhibitors for advanced stage solid malignancies. The racial diversity of the population of patients is a unique feature of this study. Our data concluded a positive correlation of irAEs development with increased probability of survival and demonstrates a positive correlation with Caucasian race and overall survival.

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INTRODUCTION

Emergence of immune checkpoint inhibitors (ICPi) has revolutionized standard of care treatment in a wide spectrum of cancer subtypes resulting in increased survival[1,2]. These agents up regulate activity of the immune system by blocking intrinsic down regulators of immunity such as cytotoxic T-lymphocyte-associated protein 4 and programmed cell death-protein 1 (PD-1)[1]. This amplified immune activity can also result in off target autoimmune inflammation of healthy organs resulting in immune-related adverse effects (irAE)[1,3].

Various mechanisms contribute to the irAE affecting several organ systems, with effect on the endocrine glands noted as one of the most common and often irreversible

irAE[1,4-7]. Correlation with the development of these adverse effects on outcomes is controversial, however, there is a growing body of evidence that the development of these adverse events may be associated with improved outcomes[1,8-13].

In this retrospective analysis, we aim to describe the association of development of irAEs to survival outcomes in patients treated with ICPi for advanced stage solid malignancies. We also specifically underscore the effects of endocrine irAEs on survival. More importantly, we sought to address differences in survival outcomes across different racial groups given the significant underrepresentation of minority racial groups in pivotal immunotherapy trials[14].

We hypothesize that development of irAEs will have a positive correlation with progression free survival (PFS) and overall survival (OS) in patients treated with ICPi. We also anticipate that there will be differences in outcomes among various racial groups. There is very limited data on efficacy outcomes in racially diverse patients who receive immunotherapy and develop irAEs making our study unique and clinically relevant.

MATERIALS AND METHODS

This is a retrospective analysis of patients with a diagnosis of stage IV solid malignancy who were treated with PD-1 or programmed death ligand 1 (PDL1) blocker between January 1, 2013 and December 31, 2018 across MedStar Georgetown Cancer Institute facilities. This study was approved by the MedStar institutional review board and a waiver of HIPAA authorization was obtained given its retrospective nature.

Patients

All patients with stage IV solid malignancy in follow-up from January 1, 2013 to December 31, 2018 across MedStar Georgetown Cancer Institute facilities were identified using ICD-10 diagnostic codes. The electronic medical records were reviewed to identify patients who have been initiated on an ICPi with pembrolizumab, atezolizumab, durvalumab, or nivolumab. Patients with hematological malignancies, early-stage solid malignancies and those treated in combination with cytotoxic T-lymphocyte-associated protein 4 inhibitors, such as ipilimumab, were excluded.

Pertinent data collected from the records included age at the time of ICPi initiation, gender, race which was self-identified by patients, date of metastatic disease diagnosis, type of cancer, sites of metastasis, line of treatment, molecular markers, percentage of PDL1 expression, date of start of ICPi, date of first reported irAE, type of irAE, severity of irAE based on Common Terminology Criteria for Adverse Events Version 5.0[15], systemic steroid use, date of death or date of last follow up.

Endpoints

Differences in median PFS and OS and probability of survival among patients who had irAE *vs* those without irAE were primary endpoints. Data were further stratified based on age, gender, race, PDL1 expression, line of therapy, median number of cycles and LDH. Secondary endpoints included difference in median PFS and OS and probability of survival among those with endocrine irAE. PFS was defined as the time from initiation of ICPi therapy to radiographic progression. OS was defined as the time from initiation of ICPi therapy to date of death or last follow up.

Statistics

Descriptive statistics were used to summarize demographics, PDL1 expression, line of therapy, number of cycles of immunotherapy. Continuous variables were described by means with standard deviations and medians and first and third quartiles. Two-sample *t*-test was used to examine differences in the averages between the groups if normality assumption was satisfied and the non-parametric Wilcoxon rank sum test was used when normality assumption was not satisfied. Categorical variables were described by frequencies and column percentages, and Chi-square and Fisher exact tests as appropriate were used to investigate differences between the groups. Kaplan-Meier curves were provided for overall and progression free survival and log rank test was used to compare the curves between the two groups. Statistical significance was defined as $P < 0.05$.

RESULTS

Patients

464 patients with stage IV solid malignancies were selected for review, and 293 patients met eligibility criteria. 91 patients (31%) had any grade irAE; most common AE were endocrine (40.7%), specifically TSH elevation, dermatological (23.1%) and rheumatologic (18.7%). Patient characteristics and incidence of irAE are described in detail in Tables 1-3. Proportion of irAE was significantly higher in Caucasians *vs* African Americans (AA) at 60.4% and 30.8% respectively (P = 0.01). Mean age of patients at initiation of ICPi was 63 years ± 12.8. The incidence of irAE was significantly higher in older patients, with a mean age of 66 years ± 13.2 in comparison to a mean age of 62 years ± 12.4 in those without irAE (P = 0.0108). PDL1 positivity, defined as greater than or equal to 1%, was observed in 71 patients while PDL1 negativity was observed in 70 patients; 192 patients had missing PDL1 status. The incidence of irAE did not differ between the two groups (P = 0.07). However, when these patients were further stratified to PDL1 status < 50% (n = 69) or ≥ 50% (n = 32), there was a statistically significant increase in incidence of irAE in the low PDL1 positive group at 17.6% *vs* 15.4% (P = 0.03). Statistically significant relationship was found between number of cycles of immunotherapy and irAE. The median number of cycles of immunotherapy in patients who had irAE is higher than patients who had no irAE at 8 and 4 respectively (P = 0.001). Median LDH was also lower at 190 in patients with irAE *vs* those who did not at 253 (P = 0.04).

Primary endpoints

Rate of progression was lower in patients with irAE at 30.8% *vs* 46.0%, (P = 0.01). Median PFS was higher at 5.8 mo in patients with irAE *vs* those without at 3.0 mo (P = 0.02). Median OS was also higher in those with irAE *vs* no irAE at 17.1 and 7.2 mo respectively (P < 0.0001). The probability of PFS and OS was statistically significant (P = 0.0088 and P = 0.0004), respectively, based on Kaplan-Meier curves (Figure 1). Statistical significance between groups was defined as P < 0.05.

Secondary endpoints

Statistically significant difference in median OS (17.1 *vs* 8.6 mo, P = 0.0002) but not in PFS (5.8 *vs* 3.3 mo, P = 0.0545) was noted with endocrine irAE based on Wilcoxon rank sum test. However, these differences were not seen on Kaplan-Meier analysis (Figure 2). No differences in survival were observed among other commonly reported irAE. Differences in survival among subgroups of patients with irAE and endocrine irAE are detailed in Table 4. Median OS was higher with Caucasian race *vs* AA in patients with irAE (20.6 *vs* 12.9 mo, P = 0.0237) and in those who specifically had endocrine irAEs (21.8 *vs* 15.8 mo, P = 0.0356). However, once again these did not translate to better survival probability on Kaplan-Meijer analysis (Figure 3).

DISCUSSION

Development of irAE in patients treated with ICPi is not uncommon and is an off-target effect of increased immune activity[1]. Several studies done in specific tumor types using various anti-PD-1 and anti-PDL1 agents confirmed the association of irAE development with improved outcomes[8-12,16]. Our study similarly concluded a positive correlation of irAE development with increased probability of survival. The inclusion of a racially diverse population of patients in our study is unique and has not been previously reported to the best of our knowledge. Moreover, we also noted important associations with development of endocrine irAE with survival outcomes.

Median PFS and OS were significantly improved in patients who developed irAE in our study. Moreover, positive correlation in median OS was also seen in patients who had endocrine adverse events. A study by Grangeon *et al*[17] assessing similar survival endpoints in patients with advanced stage non-small cell lung cancer (NSCLC) concluded improved objective response rates, PFS and OS in patients who received ICPi therapy. Moreover, in patients who had thyroid dysfunction PFS and OS were significantly improved. Other studies reported similar outcomes in patients with NSCLC who had thyroid AE following treatment with immunotherapy[9,10,18]. A preliminary study from our group also addressed this issue with a systematic literature review and meta-analysis of patients with lung cancer and head and neck cancer on immune checkpoint inhibitor therapy across twelve randomized-controlled trials comprising of 7060 patients with 3815 patients using ICPi[19]. In that study, at a

Table 1 Patient characteristics

	irAE						
	Overall		Yes		No		
	n	%	*n*	%	*n*	%	
Gender							0.8120
Female	129	44.0	41	45.1	88	43.6	
Male	164	56.0	50	54.9	114	56.4	
Race							0.0141
White	140	47.8	55	60.4	85	42.1	
Black	122	41.6	28	30.8	94	46.5	
Asian	13	4.4	4	4.4	9	4.5	
Hispanic	2	0.7	0	0	2	1.0	
Native American	1	0.3	1	1.1	0	0	
Other	11	3.8	1	1.1	10	5.0	
Unknown	4	1.4	2	2.2	2	1.0	
Death							0.1488
No	131	44.7	35	38.5	96	47.5	
Yes	162	55.3	56	61.5	106	52.5	
PDL1 positive							0.0695
Yes	71	24.2	25	27.5	46	22.8	
No	70	23.9	15	16.5	55	27.2	
Missing	152	51.9	51	56.0	101	50.0	
PDL1 expression							0.0354
< 50%	69	23.5	16	17.6	53	26.2	
≥ 50%	32	10.9	14	15.4	18	8.9	
Missing	192	65.5	61	67.0	131	64.9	
Line of therapy							0.9432
1	85	29.0	29	31.9	56	27.7	
2	130	44.4	37	40.7	93	46.0	
3	35	11.9	12	13.2	23	11.4	
4	17	5.8	5	5.5	12	5.9	
5	8	2.7	2	2.2	6	3.0	
6	2	0.7	1	1.1	1	0.5	
7	2	0.7	0	0	2	1.0	
8	1	0.3	0	0	1	0.5	
Missing	13	4.4	5	5.5	8	4.0	
Progression							0.0140
Yes	121	41.3	28	30.8	93	46.0	
No	172	58.7	63	69.2	109	54.0	

irAE: Immune-related adverse events; PDL1: Programmed death ligand 1.

mean follow-up of 12.2 mo, the OS (hazard ratio: 0.75; 95% credibility interval: 0.70-0.80) and PFS (hazard ratio: 0.77; 95% credibility interval: 0.72-0.81) in the ICPi arm were significantly improved. Endocrine irAE, specifically hypothyroidism, was the most common adverse event with a prevalence of 9% (P = 0.019). However, none of these studies accounted for differences among various racial groups and outcomes in all cancer subtypes is sparse.

Minority patient enrollment, specifically African American patients, in immunotherapy trials across various tumor types was less than 5%[14]. This raises an important gap in the understanding of clinical outcomes in these patients with the use of ICPi. A study done in 136 metastatic NSCLC patients who received immunotherapy at our institutions did not show a difference in efficacy or safety among Caucasians and AA races (unpublished results). Out of the 293 patients in our study with various stage IV solid malignancies, 41.6% of patients were AA. The incidence of irAE was significantly higher in Caucasians *vs* AA 60.4% and 30.8% respectively, P = 0.0141. Moreover, a higher median OS was also seen with Caucasian race *vs* AA in patients with irAE (20.6 *vs* 12.9 mo, P = 0.0237) and in those with endocrine irAEs (21.8 *vs* 15.8 mo, P = 0.0356). Although this did not translate to a better probability of survival, our study adds important real-world data in this under-represented group of patients.

A major limitation to our study is the significant heterogeneity in baseline patient characteristics including tumor subtype, type of immunotherapy used, and line of treatment. Despite these limitations, our study confirmed our primary endpoint of improved survival outcomes in patients who develop irAE during treatment. In other subgroups of interest such as Caucasian race and patients with endocrine irAE, there was a positive correlation with median OS however this was not translated to increased rate of survival due to discordant results between the two statistical tests. Prospective studies in similar patients with pre-specified multivariate analyses accounting for tumor type, type of ICPi and line of treatment may further elicit important patterns and could lead to definitive conclusions in these subgroups.

CONCLUSION

Development of irAEs improves PFS and OS in patients with advanced stage solid malignancies treated with ICPi. Our study also underscored two important factors, race and endocrine irAEs, as possible surrogate markers for improved survival given the noted positive correlation with median overall survival.

Table 2 Patient characteristics

	irAE									
	Overall			Yes			No			*P* value
	n	mean	SD	*n*	mean	SD	*n*	mean	SD	
Age at ICPi initiation	293	63.3	12.8	91	66.1	13.2	202	62.0	12.4	0.0108

irAE: Immune-related adverse events; ICPi: Immune checkpoint inhibitors.

Table 3 Patient characteristics

	irAE									
	Overall			Yes			No			*P* value
	n	Median	(Q1, Q3)	*n*	Median	(Q1, Q3)	*n*	Median	(Q1, Q3)	
Number of cycles of immunotherapy	277	5	(2, 12)	82	8.0	(3.0, 19.0)	195	4.0	(2.0, 9.0)	0.0011
LDH	55	243	(171, 290)	25	190	(163, 259)	30	253	(186, 383)	0.0376
Progression free survival (mo)	118	3.9	(1.9, 7.4)	27	5.8	(2.7, 13.0)	91	3.0	(1.7, 6.8)	0.0204
Overall survival (mo)	288	11.5	(2.9, 19.4)	89	17.1	(8.4, 23.5)	199	7.2	(2.1, 15.3)	< 0.0001

irAE: Immune-related adverse events.

Table 4 Progression free survival and overall survival calculated using Wilcoxon rank sum test for comparison

		Overall, *n* (%)	irAE, *n* (%)	irAE, Median PFS (mo)	*P* value	irAE, Median OS (mo)	*P* value	Endo irAE, *n* (%)	Endo irAE, Median PFS (mo)	*P* value	Endo irAE, Median OS (mo)	*P* value
Gender	Female	129 (44)	41 (45.1)	6.3	0.8977	16.3	0.4756	18 (48.7)	7.7	0.8542	17.0	0.9495
	Male	164 (56)	50 (54.9)	5.7		18.5		19 (51.4)	5.7		17.0	
Race	White	140 (47.8)	55 (60.4)	7.7	0.4601	20.6	0.0237	23 (62.2)	10.4	0.2243	21.8	0.0356
	Black	122 (41.6)	28 (30.8)	5.8		12.9		13 (35.1)	5.2		15.8	
	Others	31 (10.6)	8(8.8)	2.2		16.0		1 (2.7)			6.2	
PDL1 Expression	< 50%	69 (23.5)	16 (17.6)	5.2	0.6985	14.2	0.6468	3 (25.0)	5.2	1.000	16.5	1.0000
	> 50%	32 (10.9)	14 (15.4)	6.3		16.6		9 (75.0)	6.3		15.8	

PFS: Progression free survival; OS: Overall survival; irAE: Immune-related adverse events; PDL1: Programmed death ligand 1.

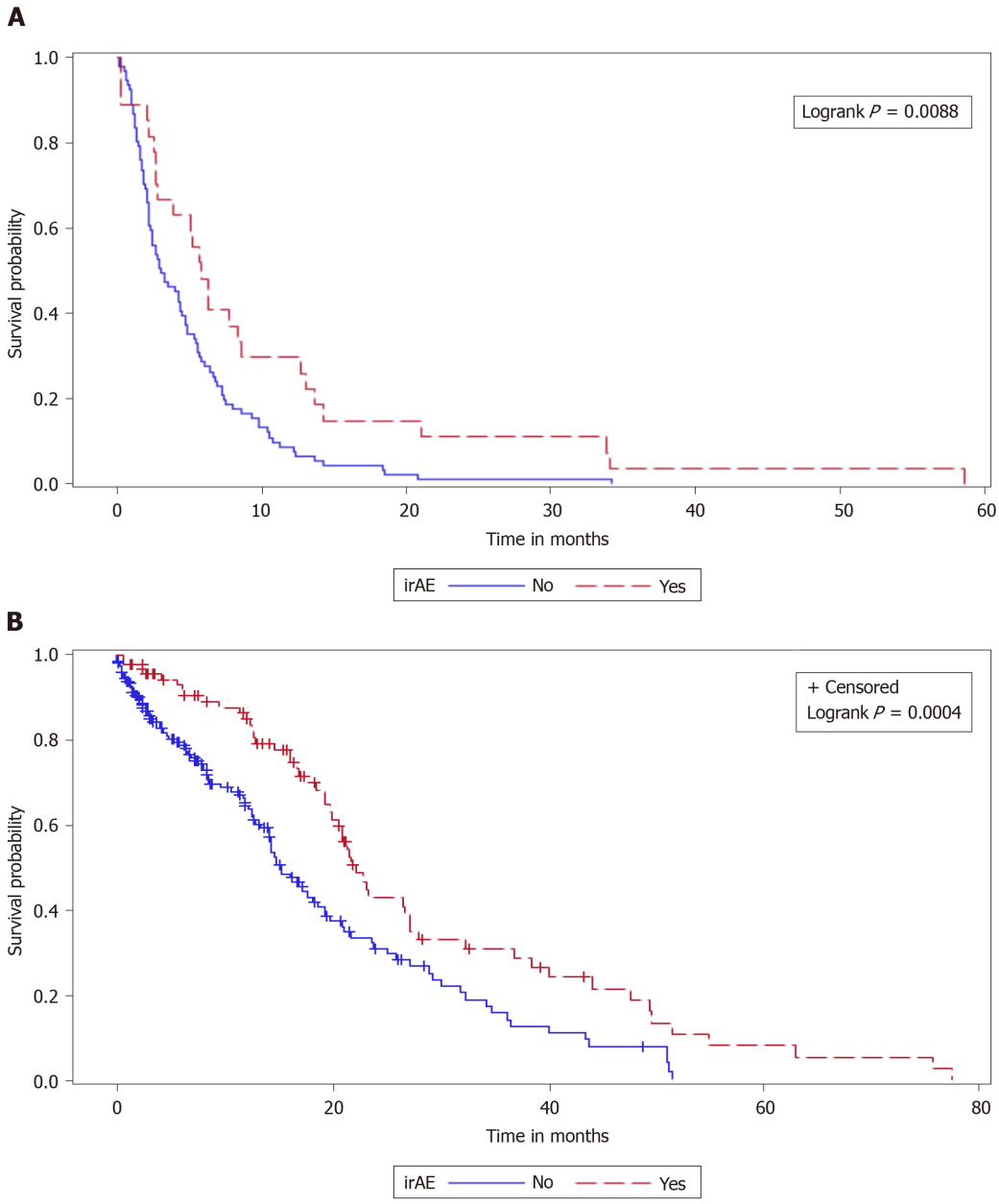


Figure 1 Kaplan-Meier curves. Progression free survival (A) and overall survival (B) of immune-related adverse events *vs* no immune-related adverse events using Kaplan-Meier methods with log-rank test for *P* value. irAE: Immune-related adverse events.

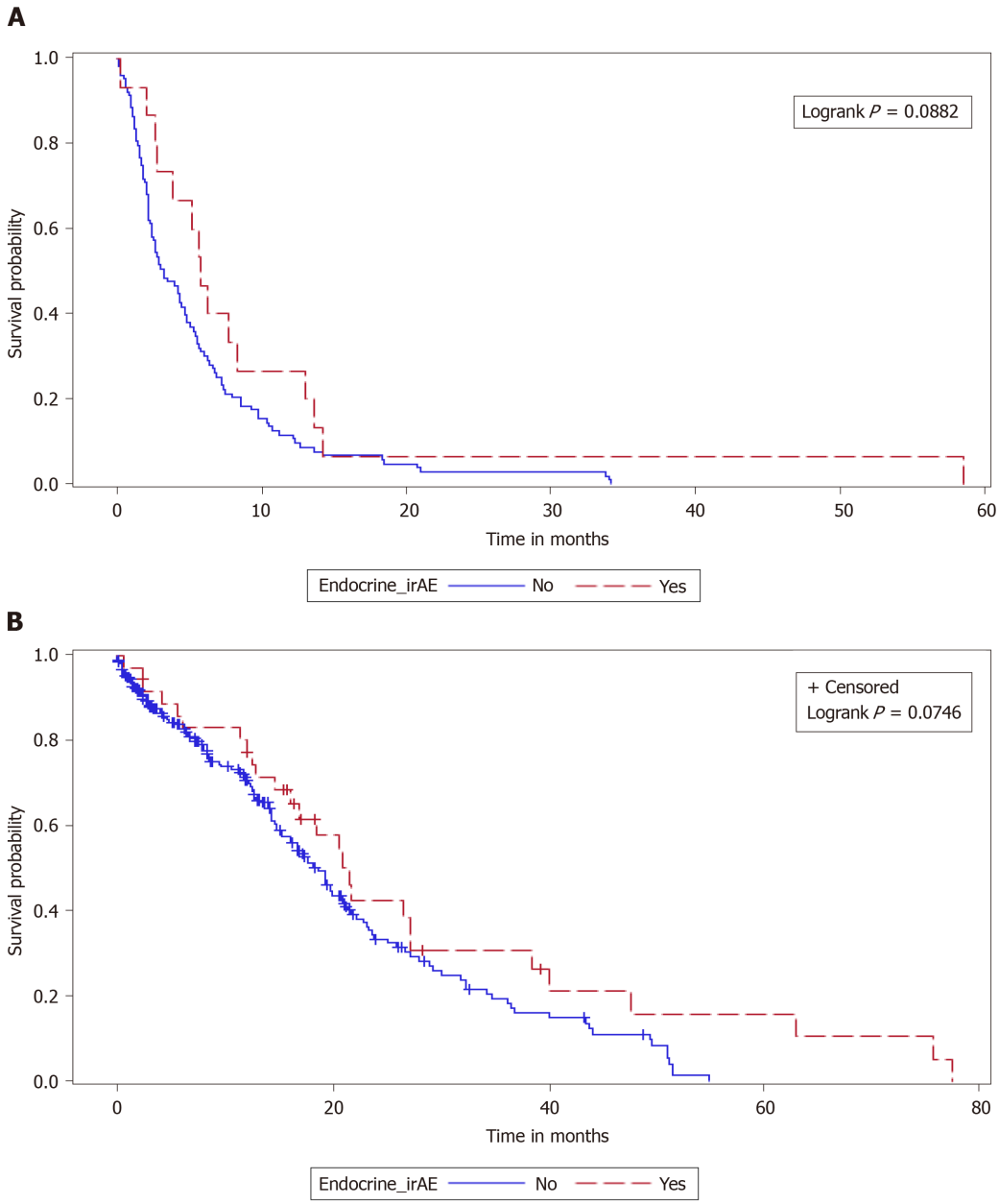


Figure 2 These differences were not seen on Kaplan-Meier analysis. Progression free survival (A) and overall survival (B) of endocrine immune-related adverse events *vs* no endocrine immune-related adverse events using Kaplan-Meier methods. irAE: Immune-related adverse events.

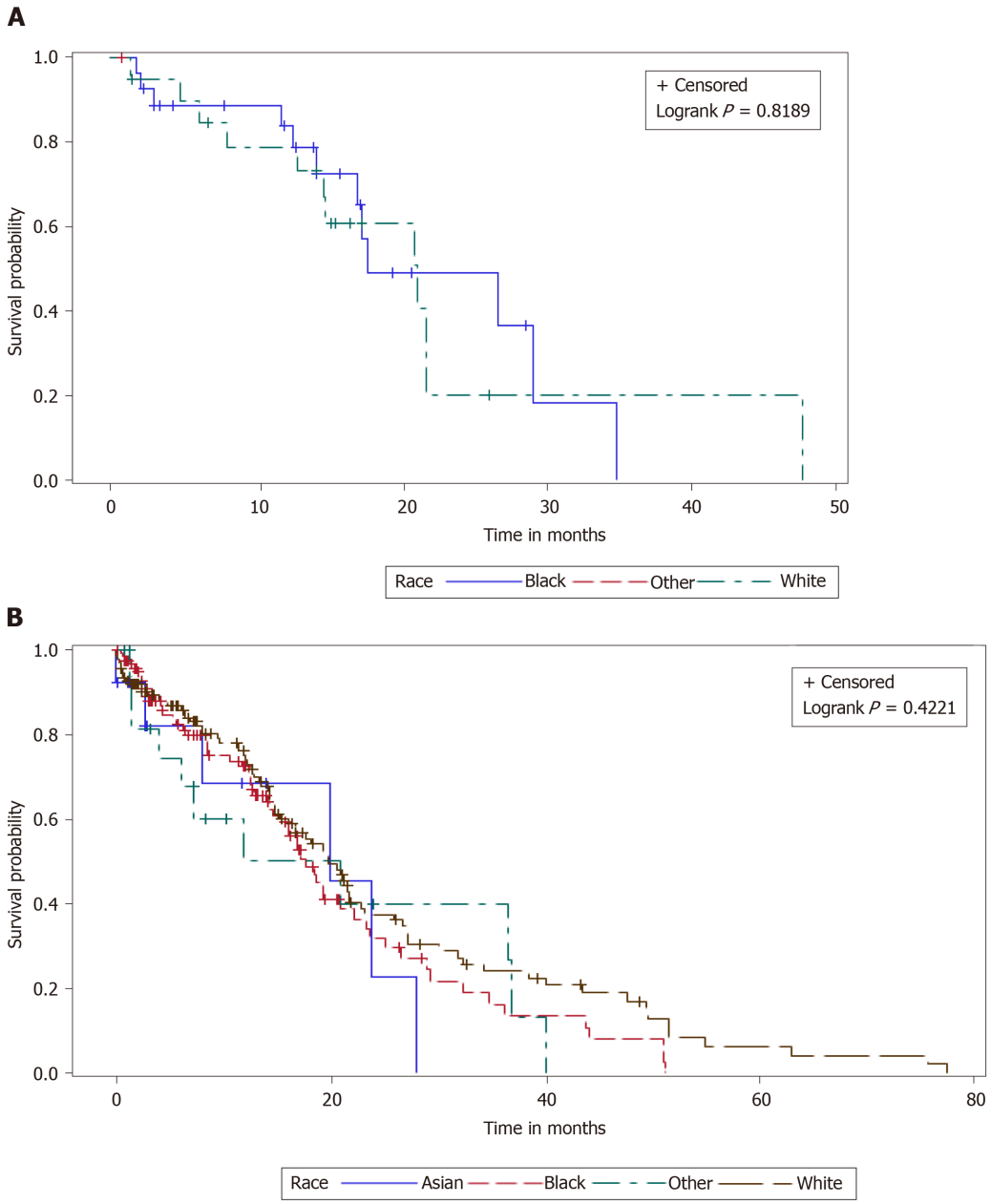


Figure 3 These did not translate to better survival probability on Kaplan-Meijer analysis. Progression free survival (A) and overall survival (B) based on race using Kaplan-Meier methods.

ARTICLE HIGHLIGHTS

Research background

Immune checkpoint inhibitors (ICPi) constitute a revolutionary therapy for various types of cancer. However, their use is associated with the development of several immune-related adverse events (irAEs), especially related to the endocrine system.

Research motivation

Recently, some studies have suggested that the development of irAEs could result in improved survival outcome.

Research objectives

This retrospective study aimed to describe the association of development of irAEs to survival outcomes in patients treated with ICPi for advanced stage solid malignancies. Moreover, we investigated the effects of endocrine irAEs on survival and we addressed differences in survival outcomes among different racial groups.

Research methods

This is a retrospective analysis of data on 293 patients with diagnosis of stage IV solid malignancy who were treated with programmed cell death-protein 1 or programmed death ligand 1 blocker between 1 January 2013 and 31 December 2018. Progression free survival and overall survival were primary endpoints.

Research results

Out of 293 patients, 91 (31%) had any grade irAEs, amongst which the most common were endocrine (40.7%), followed by dermatological (23.1%) and rheumatologic (18.7%). Caucasians had more irAEs than African Americans (60.4% *vs* 30.8%). Patients with irAE had a lower rate of progression (30.8% *vs* 46.0%, P = 0.0140). Median progression free survival was higher in patients with irAE *vs* those without (5.8 *vs* 3.0 mo respectively, P = 0.02). Similarly, median overall survival was higher in those with irAE *vs* no irAE (17.1 *vs* 7.2 mo respectively, P < 0.0001).

Research conclusions

This study demonstrates that the development of irAEs is positively correlated with improved survival. It also demonstrates a positive correlation with Caucasian race and overall survival.

Research perspectives

Future prospective studies designed to evaluate cofounding variables for survival such as type of ICPi used, tumor type, and line of treatment are needed to draw definitive conclusions.

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GUIDELINES

GOECP/SEOR radiotherapy guidelines for small-cell lung cancer

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Abstract

Small cell lung cancer (SCLC) accounts for approximately 20% of all lung cancers.

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The main treatment is chemotherapy (Ch). However, the addition of radiotherapy significantly improves overall survival (OS) in patients with non-metastatic SCLC and in those with metastatic SCLC who respond to Ch. Prophylactic cranial irradiation reduces the risk of brain metastases and improves OS in both metastatic and non-metastatic patients. The 5-year OS rate in patients with limited-stage disease (non-metastatic) is slightly higher than 30%, but less than 5% in patients with extensive-stage disease (metastatic). The present clinical guidelines were developed by Spanish radiation oncologists on behalf of the Oncologic Group for the Study of Lung Cancer/Spanish Society of Radiation Oncology to provide a current review of the diagnosis, planning, and treatment of SCLC. These guidelines emphasise treatment fields, radiation techniques, fractionation, concomitant treatment, and the optimal timing of Ch and radiotherapy. Finally, we discuss the main indications for reirradiation in local recurrence.

Key Words: Small cell lung cancer; Chemotherapy; Hyperfractionated radiation therapy; Prophylactic brain irradiation; Brain metastases; Reirradiation

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Core Tip: Small cell lung cancer is the paradigmatic model of multidisciplinary cancer treatment. The improvement in overall survival over the last 70 years is primarily due to combined therapies and close collaboration among specialists to administer local and systemic therapies. This cooperation has enabled clinicians to achieve an additive effect by combining chemotherapy and radiotherapy while ensuring that the overall toxicity is manageable. The present review describes the evolution of the treatment of small cell lung cancer in recent decades as well as continuing advances in the multimodal therapeutic management of this disease.

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INTRODUCTION

Evolution of the multimodal treatment of small-cell lung cancer

Small cell lung cancer (SCLC) was first described by Barnard[1] in 1926. Prior to that time, SCLC was considered a mediastinal lymphosarcoma and later it was known as "oat cell carcinoma of the bronchus"[2]. The treatment of SCLC has evolved over time and this evolution is reflected in published randomised controlled trials (RCT). Table 1 summarizes the main clinical trials carried out to date to evaluate the treatment of SCLC. Based on advances in thoracic surgery during World War II, surgery became the treatment of choice in SCLC until 1960[3].

The first RCT comparing surgery to radiotherapy (RT) in SCLC was performed in the 1960s[4,5]. After that, the Veterans Administration Lung Study Group (VALSG) defined SCLC as either limited-stage (LS) or extensive-stage (ES) disease depending on whether or not the disease could be encompassed within a single RT field[6].

Later, a RCT comparing cyclophosphamide (CP) to placebo found that CP significantly improved overall survival (OS)[7]. In a subsequent clinical trial, combined therapy (RT + CP) increased survival *vs* RT alone[8]. Other RCTs later confirmed those findings[9,10], while the findings of a subsequent RCT conducted by Perez *et al*[11] suggested that prophylactic cranial irradiation (PCI) could further prolong survival.

Polychemotherapy (PCh) regimens were evaluated in various clinical trials[12-14], one of which concluded that PCh was superior to CP[15]. As a result, RT was relegated to being used as consolidation therapy[16,17]. Other studies found that combining

Table 1 Chronological changes in the combined treatment of small cell lung cancer

Chronological changes in SCLC treatment							
Ref.	**Stage (LS or ES)**	**Phase**	**Treatment**	**Median survival**	**5-yr survival**	***P* value**	**Level of evidence**
Miller *et al*[4] 1969	LS	III	RT	28.5 wk; 43 wk	1%; 5%	0.04	A I
Bergsagel *et al*[8] 1972	LS	III	RT; RT + Ch	21 wk; 42 wk	NR	< 0.05	A I
Einhorn *et al*[13] 1976	LS	II	RT + PCT	12 mo	10%		C III
Bunn *et al*[18] 1987	LS	III	PCT; PCT + RT	12 mo; 15 mo	10%; 15%	< 0.05	A I
Turrisi *et al*[27] 1988	LS	II	Early AHF-RT + CE	23 mo	30% at 3 yr		C III
Murray *et al*[26] 1993	LS	III	Early RT + CE; late RT + CE	21 mo; 19 mo	20%; 13%	< 0.05	A I
Pignon *et al*[21] 1992	LS	Meta-analysis	PCT (no CE); PCT (no CE) + TRT	< 14% mortality	> 5% at 3 yr	0.001	A I
Jeremic *et al*[29] 1997	LS	III	Early AHF-RT + CE; late AHF-RT + CE	36 mo; 34 mo	30%; 15%	0.0027	A I
Turrisi *et al*[31] 1999	LS	III	Early AHF-RT + CE; early NFRT + CE	23 mo; 19 mo	26%; 16%	0.04	A I
Jeremic *et al*[41] 1999	ES	III	PCT + RT + PCI; PCT + PCI	17 mo; 11 mo	9.1%; 3.7%	0.0041	A I
Aupérin *et al*[33] 1999	LS	Meta-analysis	PCI; no PCI	> 6% at 3 yr			A I
Takada *et al*[30] 2002	LS	III	Early AHF-RT + CE; late AHF-RT + CE	31.3 mo; 20.8 mo	24%; 18%	< 0.05	A I
Slotman *et al*[38] 2007	ES	III	CE + PCI; CE		27% at 1 yr; 13% at 1 yr	< 0.001	A I
Slotman *et al*[42] 2015	ES	III	CE + PCI; CE + TRT + PCI		3% at 3 yr; 13% at 3 yr	< 0.03	A I
Faivre-Finn *et al*[43] 2017	LS	III	CE + AHF-RT 45 Gy; CE + NFRT 66 Gy	29 mo; 19 mo	34%; 31%	NS	A I

LS: Limited-stage; ES: Extensive-stage; TRT: Thoracic radiotherapy; AHF-RT: Accelerated hyperfractionated radiotherapy; NFRT: Normofractionated radiotherapy; Ch: Chemotherapy; PCT: Polychemotherapy; PCI: Prophylactic cranial irradiation; CE: Cisplatin + etoposide; NS: Not significant.

chemotherapy (Ch) and RT (chemoradiotherapy; CRT) resulted in median survival outcomes of 12-15 mo and 5-year OS rates ranging from 10%-15%. While CRT seemed to yield comparable OS outcomes, treatment-related toxicity was substantially greater when the two treatment modalities were combined[18,19]. Two meta-analyses (MTA) found that adding RT to Ch improved 3-year OS[20-22].

Based on previous experience with PCh regimens, a treatment protocol involving RT to the brain and thorax combined with high dose Ch [CP, Adriamycin and Vincristine (CAV)] was developed, achieving greater local control (LC) than either treatment alone[23]. However, that approach was abandoned due to high levels of treatment-related toxicity[24]. Cisplatin-etoposide Ch combined with RT has shown to be effective in the treatment of SCLC with a manageable toxicity profile[25].

The next advance in treatment protocols for LS-SCLC came from a RCT comparing early thoracic RT (TRT) to late TRT, both groups received concurrent PCh, showing a significant benefit in 5-year OS for early TRT[26]. A phase II trial compared once-daily TRT to twice-daily (1.5 Gy/session) accelerated hyperfractionated RT (AHF-RT) (45 Gy). All patients received concurrent cisplatin-etoposide PCh[27]. The median survival in the AHF-RT group was 23 mo, with a 3-year OS of 30%[28].

Another RCT[29] was performed to compare initial (weeks 1 to 4) to delayed (weeks 6-9) AHF-RT (54 Gy), with all patients receiving concurrent low-dose PCh. Median survival in the early group was 34 mo *vs* 26 mo in the delayed group; 5-year OS was 30% *vs* 15%, respectively (P = 0.027). Another RCT[30] compared AHF-RT combined with cisplatin-etoposide administered concurrently or sequentially. The results of that trial showed that concurrent CRT was associated with significantly better OS outcomes

($P < 0.05$) than sequential treatment. Later, another phase III trial[31] compared concurrent AHF-RT to normofractionated TRT, with both groups also receiving adjuvant cisplatin-etoposide PCh. OS was significantly better in the AHF-RT group ($P = 0.04$). Nevertheless, this OS benefit was not confirmed in a subsequent RCT comparing normofractionated RT to AHF-RT[32]. A MTA found that patients with LS-SCLC who underwent PCI after achieving complete remission had a 6% improvement in 3-year OS[33].

The so-called "Turrisi scheme" (AHF-RT) became controversial when critics rejected this treatment approach for the following reasons: (1) The TRT doses in the accelerated and normofractionated regimens are not biologically-equivalent; (2) This scheme implies higher treatment-related costs for the health care system; (3) Twice-daily RT means important organizational challenges; and (4) AHF-RT is associated with higher rates of esophageal toxicity. Furthermore, high-dose, normofractionated regimens seem to show equivalent results in terms of OS[34] and some MTA have found contradictory results[35,36].

Advances in SCLC staging, PCI in stage IV disease

Two important studies were published in 2007: The first proposed staging SCLC according to the AJCC (American Joint Committee on Cancer) TNM (tumor-node-metastasis) criteria[37]. The second study reported improved OS outcomes in patients with ES-SCLC who had a confirmed response to PCh and subsequently received PCI[38]. Based on those findings, PCI became the standard treatment for patients with LS-SCLC who showed a partial response (PR) to treatment.

Regarding diagnostic techniques, 18^{F}-fluorodeoxyglucose (FDG) positron-emission tomography-computed tomography (PET/CT) was incorporated into clinical practice because it provides more accurate staging of supraclavicular and distant disease, thus reducing the need for prophylactic mediastinal irradiation[39,40].

Phase III trials: CREST for stage IV disease and CONVERT for stages I-III

In recent years, the results of two landmark RCT were published: CREST and CONVERT[41-43]. CREST was performed to evaluate consolidative TRT in patients with ES-SCLC who respond to Ch. Median survival was significantly longer in the treatment group with better 5-year OS rates. In the CREST trial[41,42], patients were randomised to consolidative TRT (30 Gy in 10 fractions) or no TRT (control group). All patients received PCI. Thoracic recurrences were significantly reduced in the intervention group *vs* control (80% *vs* 44%). The 2-year OS was 13% *vs* 3%. A post hoc analysis revealed a greater survival benefit in patients who underwent thoracic RT after PCh[44] and in patients with ≤ 3 extrapulmonary metastases who received consolidative TRT[45].

In the CONVERT trial[43], patients were randomised to twice-daily AHF-RT (45 Gy) *vs* once-daily RT (66 Gy). Both groups received PCI. There were no significant differences between the groups in OS, progression-free survival (PFS), LC, or metastatic progression. Based on these findings, the Turrisi scheme remained the treatment of choice due to higher compliance rates (98% *vs* 83%) with the TRT dose. A subsequent MTA showed that OS was better in patients treated with early *vs* late TRT in patients who complied with the prescribed dose intensity of the Ch regimen[46].

Consolidation RT for metastatic lesions in stage IV SCLC

Consolidation immunotherapy in ES-SCLC: In a RCT, patients with stage IV ES-SCLC and ≤ 4 extracranial metastases after complete or partial response to PCh were randomised to PCI alone or PCI plus consolidative TRT for intrathoracic disease and extracranial metastases. The trial was closed at the interim analysis due to slow recruitment and the absence of significant differences in OS[47]. The risk of first thoracic recurrence was lower in the consolidative TRT group and in patients whose metastases were irradiated.

Recently, several phase III trials have reported better survival outcomes in patients with ES-SCLC when combining carboplatin-etoposide with atezolizumab[48] or durvalumab[49,50]. The results of two RCTs comparing dose escalation with the normofractionated scheme *vs* the "Turrisi" scheme are pending: CALGB 30610 (normofractionation to 70 Gy) and a Norwegian study involving high-dose (60 Gy) AHF-RT in the experimental arm[51].

DIAGNOSIS, EVALUATION OF DISEASE SPREAD AND STAGING OF SCLC

SCLC is commonly diagnosed in advanced stages. Early detection of SCLC with computed tomography has not shown to provide any benefit. Given the aggressive nature of advanced SCLC, the diagnosis should be made in a few weeks, but treatment should not be initiated until histological confirmation[52] is done.

In patients with SCLC, the following key variables should be registered: Age, comorbidities, tobacco use, and ECOG status. Patients should undergo a complete physical examination with comprehensive blood tests. Molecular markers provide little value, although pro-gastrin-releasing peptide has shown specificity in the diagnosis and follow-up of some patients with SCLC[53].

Paraneoplastic syndromes can occur due to the production of polypeptides (syndrome of inappropriate antidiuretic hormone secretion or Cushing's syndrome), or autoimmune phenomena (Lambert-Eaton syndrome, anti-Hu-associated encephalomyelitis, among others).

Staging is performed *via* thoracoabdominal computed tomography with intravenous contrast (IVC), unless contraindicated. Preferably, magnetic resonance imaging (MRI) should be used to perform the brain study due to its greater sensitivity and better definition of brain structures. Brain metastases (BM) are present in more than 12% of patients previously classified with LS-SCLC.

^{18}F-FDG PET/CT imaging is recommended in LS-SCLC, but considered optional in ES-SCLC if it implies a delay in treatment. This imaging modality provides the best mediastinal staging and distant localization of metastases (adrenal and bone)[54]. Due to its high sensitivity (close to 100%) and specificity (90%), ^{18}F-FDG PET/CT imaging changes the therapeutic approach in 28% of patients[55], including re-staging in 15% of cases [95% confidence interval (CI): 9-21][56].

^{18}F-FDG PET/CT images should be acquired under the same positioning and immobilization conditions of the planned treatment. This imaging modality can help to optimize radiation doses and treatment volumes[57]. One study reported a higher risk of supraclavicular lymph node (LN) recurrence when CT-based nodal irradiation was omitted[58]. It has been suggested that better definition of volumes could influence both LC and OS[59]. Although this improvement in OS has not been demonstrated in prospective studies[60], some have reported lower rates of esophagitis[57]. In patients who undergo sequential treatment, Ch can reduce glucose consumption; consequently, restaging with ^{18}F-FDG PET/CT may underestimate mediastinal involvement, which is why this imaging modality is not appropriate for planning RT[61,62].

Bony metastases are often present in these patients. A bone scan is indicated if ^{18}F-FDG PET/CT is not available[63]. If there is suspected bone marrow involvement, a biopsy is indicated (level of evidence V, grade of recommendation C)[64]. Abdominal MRI with IVC may be necessary to identify hepatic or adrenal lesions (level of evidence V, grade of recommendation C).

Histologic confirmation is generally obtained through bronchoscopy, endobronchial ultrasound (EBUS), or transesophageal endoscopic ultrasound (level of evidence III, grade of recommendation C)[65]. EBUS-guided needle aspiration is highly effective, with a sensitivity of 97.4%, specificity of 100%, negative predictive value of 60%, and positive predictive value of 100%[66]. Peripheral nodal biopsy, mediastinoscopy, or diagnostic thoracoscopy will rarely be necessary. In patients with ES-SCLC, it is recommended to biopsy the soft tissue lesions that offer the highest yield with the least risk (*e.g.*, skin, liver). Prior to surgery or RT, all patients should undergo respiratory function testing. Table 2 summarizes the imaging tests recommended for staging SCLC.

According to the World Health Organization[67], SCLC is an epithelial neoplasm of neuroendocrine origin, with small cells on Alcian blue staining, scant cytoplasm, dispersed chromatin, inconspicuous nucleoli, numerous mitoses (> 10/2 mm^2) and abundant necrosis. These lesions may be found as well in adenocarcinoma cells or squamous cell carcinoma. Immunohistochemistry will clarify diagnostic uncertainty based on expression of chromogranin, synaptophysin, and CD56, although these markers may be absent in 5%-10% of cases. CK7 is present in 25% and TTF-1 in 85%-90% of cases. Ki-67 Levels are elevated in most patients, thus facilitating differential diagnosis with other neuroendocrine tumours. Programmed death ligand 1 expression is currently being studied to determine if immunotherapy can be added to current treatment approaches. To identify new therapeutic targets, other molecular profiles can be considered in nonsmokers with ES-SCLC.

Table 2 Diagnostic staging recommendations for small cell lung cancer

Diagnosis of small cell lung carcinoma
Staging with combined VALSG and TNM AJCC 8th edition (I, A)
Baseline study
Age, tobacco use, comorbidities, complete physical examination, and ECOG PS
Complete blood analysis: Blood count, biochemistry, liver and kidney function, alkaline phosphatase, LDH
Cardiology study: Electrocardiogram +/- echocardiogram
Respiratory function testing in patients expected to receive locoregional treatment
CT with intravenous contrast (unless medically contraindicated)
Upper thoracoabdominal CT with intravenous contrast; include pelvis in advanced stages
Intravenous contrast improves the definition of central tumours and lymph node involvement (III, A)
18F-FDG PET/CT
18^{F}-FDG PET/CT recommended in patients expected to undergo locoregional treatment (III, A)
Images are acquired with the patient in the radiotherapy treatment position according to consensus protocol between Nuclear Medicine and Radiation Oncology departments (IV, A)
Not recommended for restaging after chemotherapy in sequential treatment
Brain staging
Brain MRI is preferable
Brain CT with IV contrast (without contrast is inadequate)
Bone scintigraphy
Only indicated if PET/CT is not available
Abdominal MRI
Only indicated to assess uncertain liver or adrenal lesions (V, C)
Histological confirmation
Invasive tests used as appropriate according to tumour location
Follow WHO criteria for cell typing. Immunohistochemistry for differential diagnosis

VALSG: Veterans Administration Lung Study Group; TNM: Tumor-node-metastasis; AJCC: American Joint Committee on Cancer; PS: Performance status; LDH: Lactate dehydrogenase; CT: Computed tomography; PET/CT: Positron emission tomography/computed tomography; FDG: Fluorodeoxyglucose; MRI: Magnetic resonance imaging; WHO: World Health Organization.

TNM staging

Staging for SCLC is based on the VALSG classification system. However, the TNM staging described in the AJCC 8th edition (level of evidence I, grade of recommendation A) should also be utilized[68,69]. Tables 3 and 4 summarize TNM staging for SCLC.

In patient with LS-SCLC, the tumour is confined to the hemithorax (stages I-III). All other cases of SCLC are considered ES-SCLC (stage IV). However, there are discrepancies between the various guidelines (NCCN/CSCO, ESMO) with regard to the presence of pleural effusion, large primary tumour, or contralateral supraclavicular LNs. If the only evidence of metastasis is pleural or pericardial effusion without the presence of malignant cells, these cases should be considered non-metastatic (M0) (level of evidence V, grade of recommendation B).

In patients with a single metastatic site, initiation of PCh should not be delayed. The patient should be re-evaluated after two cycles of PCh to determine the status of the target lesion (level of evidence V, grade of recommendation C).

Table 3 Tumor-node-metastasis American Joint Committee on Cancer 8th edition lung cancer

TNM AJCC 8th edition lung cancer	
T: Primary tumour	
Tx	Not evaluable by imaging or malignant cells in sputum or bronchial lavage
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	≤ 3 cm surrounded by lung or visceral pleura, or lobar bronchus
T1a (mi)	Minimally invasive
T1a	≤ 1 cm
T1b	> 1 cm to ≤ 2 cm
T1c	> 2 cm to ≤ 3 cm
T2	> 3 cm to ≤ 5 cm, or involving main bronchus without affecting the carina, visceral pleura, or atelectasis or obstructive pneumonitis extending to the hilar region, affecting part or all of the lung
T2a	> 3 cm to ≤ 4 cm
T2b	> 4 cm to ≤ 5 cm
T3	> 5 cm to ≤ 7 cm, or tumour nodules in the same lobe, or invasion of the chest wall (parietal pleura), phrenic nerve, parietal pericardium
T4	> 7 cm, or nodules in a different ipsilateral lobe or invasion of the diaphragm, mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, and carina
N: Regional lymph node involvement	
Nx	Not evaluable
N0	No node involvement
N1	Ipsilateral peribronchial and/or hilar and intrapulmonary nodes
N2	Ipsilateral mediastinal nodes and/or subcarinal
N3	Contralateral mediastinal or contralateral hilar nodes, or any scalene or supraclavicular nodes
M: Distant metastasis	
M0	No metastasis
M1	Distant metastasis
M1a	Nodules in contralateral lobe; pleural or pericardial or pleural or pericardial effusion
M1b	Single extrathoracic metastasis (including non-regional lymph node)
M1c	Multiple extrathoracic metastases

TNM: Tumor-node-metastasis; AJCC: American Joint Committee on Cancer.

CLINICAL TREATMENT INDICATIONS FOR EACH SCLC STAGE

LS disease: early stages I-IIA (T1-2N0M0)

Surgery: Patients with stage I (T1-2N0) disease may benefit from surgery[70]. These patients should undergo invasive mediastinal staging with ^{18}F-FDG PET/CT imaging[71]. Surgery consists of lobectomy with mediastinal lymphadenectomy (level of evidence II, grade of recommendation A). Based on data from retrospective studies, 5-year OS is approximately 57%[70].

Stereotactic body radiation therapy: CRT are indicated in patients with inoperable stage cT1-2N0M0 SCLC. Data from the National Cancer Data Base showed that there are no differences in OS between patients treated with stereotactic body radiation therapy (SBRT) followed by PCh and those treated with concurrent CRT[72]. As a result of those findings, the proportion of patients treated with SBRT increased sharply from 2004 (0.4%) to 2013 (6.4%). A multicenter study reported that SBRT (50 Gy, 5 fractions) yielded a LC rate of 97.4% at 1 year and 96.1% at 3 years, with a median PFS at 1 and 3 years of 58.3% and 53.2%, respectively[73]. The median survival was 17.8 mo (69.9% at 1

Table 4 Grouping by tumor-node-metastasis stage: American Joint Committee on Cancer 8th edition lung cancer

TNM AJCC 8th edition lung cancer grouping by stage			
Occult carcinoma	Tx	N0	M0
Stage 0	Tis	N0	M0
Stage IA1	T1a (mi)-T1a	N0	M0
Stage IA2	T1b	N0	M0
Stage IA3	T1c	N0	M0
Stage IB	T2a	N0	M0
Stage IIA	T2b	N0	M0
Stage IIB	T1-2	N1	M0
	T3	N0	M0
Stage IIIA	T1-2	N2	M0
	T3	N1	M0
	T4	N0-1	M0
Stage IIIB	T1-2	N3	M0
	T3-4	N2	M0
Stage IIIC	T3-4	N3	M0
Stage IVA	Any T	Any N	M1a-b
Stage IVB	Any T	Any N	M1c

TNM: Tumor-node-metastasis; AJCC: American Joint Committee on Cancer.

year and 34% at 3 years), with only minimal toxicity (grade 2 pneumonitis: 5.2%). Thus, SBRT achieves a high LC rate in patients with early, inoperable SCLC; however, sequential treatment with PCh is essential[74].

Adjuvant treatment: Adjuvant PCh should be administered after surgical resection. RT should be included in patients with mediastinal nodal involvement. Figure 1 shows the evaluation algorithm for adjuvant TRT.

A review of studies that included adjuvant TRT found that the addition of this treatment modality improved median OS in pN1 and pN2 disease[75]. Adjuvant TRT was associated with a lower risk of death in both pN1 [hazard ratio (HR): 0.79; 95%CI: 0.63-1.00, P = 0.05] and pN2 disease (HR: 0.60; 95%CI: 0.48-0.75, P < 0.0001), but not with better OS in patients without nodal involvement. OS was better (HR 0.72, 95%CI: 0.57-0.90, P = 0.004) in patients who underwent sublobar resection.

LS disease: IIB–IIIC (T3-4, N0, M0; T 1-4, N1-3, M0)

The standard treatment for stage IIB and IIIC is concomitant CRT (level of evidence I, grade of recommendation A).

Two MTA concluded that PCh plus TRT improve OS[20,21] in LS-SCLC. Another MTA[36] demonstrated that early TRT is superior to late TRT in patients receiving concomitant CRT. Ideally, TRT should start with the first or second cycle of PCh (level of evidence I, grade of recommendation A). However, PCh should not be delayed in order to initiate concomitant TRT. Another study[76] found that a shorter interval between initiation of PCh and completion of TRT is associated with a better OS (level of evidence I, grade of recommendation A).

A recent MTA[77] compared the efficacy and toxicity of twice-daily to once-daily CRT, finding significantly better overall survival with the twice-daily regimen and no differences in toxicity.

AHF-RT (45 Gy) with an interfraction interval of ≥ 6 h is recommended for normal tissue repair. Alternatively, higher dose (66 Gy) normofractionated RT can be considered as an individual option for each patient. AHF-RT is preferable in patients with a good performance status (PS) and baseline lung function.

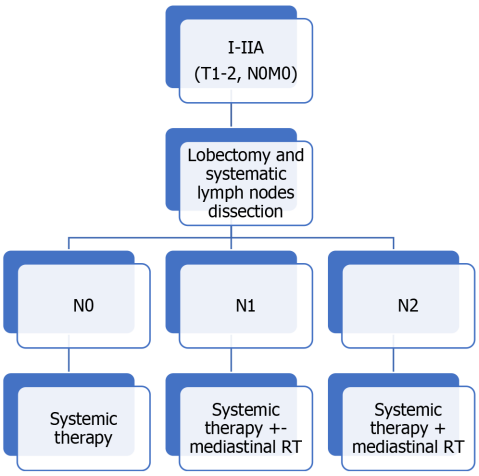


Figure 1 Therapeutic algorithm. Indications for adjuvant treatment. TNM: Tumor-node-metastasis; RT: Radiotherapy.

Extensive disease

Consolidative TRT: Prior to the introduction of immunotherapy, the main treatment for ES-SCLC was platinum-based PCh, preferably with carboplatin rather than cisplatin due to the more tolerable toxicity profile. The median OS ranges from 8-13 mo[78].

Although platinum-based PCh is the mainstay of treatment for ES-SCLC, 80% of patients present residual intrathoracic disease or progression. For this reason, consolidative TRT has been evaluated in these patients. This approach should be considered in patients with ES-SCLC who present residual thoracic disease and low volume extrathoracic metastatic disease with a complete or good response to PCh (level of evidence I, grade of recommendation A). One meta-analysis found that consolidative TRT prolongs both OS and PFS, with only a small increase in the risk of esophagitis[79].

ES-SCLC with new-onset brain metastasis (BM): BM are treated with whole-brain RT (WBRT). In asymptomatic patients, the recommended approach is PCh followed by WBRT. For symptomatic patients, the recommendation is WBRT +/- concomitant PCh.

In selected cases with only a few BM, stereotactic radiosurgery (SRS) followed by active surveillance with MRI can be considered in order to avoid PCI/WBRT; SRS can also be performed to treat new BM[80]. The FIRE SCLC study[81] was a multicenter cohort study comparing SRS to WBRT. In that study, although WBRT prolonged the interval to brain progression, it did not improve OS.

ENCEPHALON (NCT03297788) is an ongoing phase II RCT designed to compare WBRT to SRS in patients with 1–10 BM. Another trial (NCT03391362) is currently underway to compare SRS in patients diagnosed with ES-SCLC and 1-6 BM.

Oligometastatic disease: The number of metastases is a prognostic factor[82], but the differences in OS between oligometastatic and polymetastatic SCLC remain unclear. In one trial, patients with stage IV SCLC and ≤ 4 extracranial metastases after response to PCh were randomised to PCI with or without consolidative TRT and irradiation of extracranial metastases. TRT reduced the risk of the first thoracic recurrence (62.5% *vs* 25.8%) and the risk of recurrence in previous metastases (78.1% to 41.9%).

Elderly patients

The incidence of SCLC increases as a function of age, with patients ≥ age 70 accounting for approximately one-third of cases. Data on the treatment of these patients are limited because the elderly are underrepresented in clinical trials[83]. The use of CRT in elderly patients has been limited because these patients are less likely to be able to tolerate the potential toxicity of treatment due to lower functional reserve levels. However, elderly patients with LS-SCLC who are in good general condition can be treated with CRT and modern RT techniques such as 3-dimensional (3D)-RT or intensity modulated RT (IMRT). A RCT found that disease-free survival and response rates among elderly patients were similar to those observed in patients younger than age 70[84]. Even though 5-year OS was higher in younger patients, 16% of patients ≥ age 70 remained alive at 5 years. In the CONVERT trial, elderly and younger patients presented comparable OS outcomes (29 *vs* 30 mo; P = 0.38)[85]. Although many elderly

patients with LS-SCLC have comorbidities, they are generally able to tolerate CRT as well as younger patients, though they present a higher risk of asthenia and myelosuppression[86]. Patients with ES-SCLC with good PS and adequate supportive treatment can tolerate standard PCh.

DEFINITION OF VOLUMES AND ORGANS AT RISK

The ESTRO/ACROP guidelines for the target volume delineation for non-SCLC (NSCLC) categorised the recommendations as follows: Mandatory (M), recommended (R); optional (O); and discouraged (D)[87]. Some of these indications have been adopted for the treatment of SCLC[52].

Treatment planning should be based on computed tomography with the patient placed in the treatment position (M) with IVC (R) and a slice thickness ≤ 3 mm (M). Preferably, 4D-CT (R) should be used to evaluate the gross tumour volume (GTV) displacement in the respiratory cycle, or with breathing coordination methods (ABC) or gating techniques (R).

The primary GTV (GTVp) is the macroscopic tumour visible on CT or ^{18}F-FDG PET/CT. The LN GTV (GTVn) corresponds to the LNs involved prior to PCh (M) that are visible on CT or ^{18}F-FDG PET/CT (short axis > 10 mm on CT or with significant uptake), and/or have been histologically confirmed. Grouped nodes with a short axis < 10 mm adjacent to the tumour are considered to be involved (R).

The GTVp and GTVn must be contoured separately (O). The recommended lung window parameters (R) are: W = 1600 and L= -600 for lung parenchymal tumour. W = 400 and *l* = 20 for involved LNs/tumours invading the mediastinum.

At present, there are no validated data to support the fusion of diagnostic ^{18}F-FDG PET/CT with treatment planning CT[88]. There may be discrepancies between these images, for the following reasons: (1) The GTV on ^{18}F-FDG PET/CT frequently corresponds to areas of normal tissue on the CT. The ^{18}F-FDG PET/CT images are acquired over several minutes, which allows us to define the motion of the primary tumour. When these images are associated with those obtained in a 3D planning CT, some areas of the GTV on ^{18}F-FDG PET/CT may correspond to "air" areas on the CT due to lesion movement; (2) When the tumour is contiguous to areas of similar density, it must be defined by ^{18}F-FDG PET/CT; in cases in which FDG uptake is not due to the tumour (*e.g.*, heart, active infection), this should be contoured on CT; and (3) LNs that are enlarged on CT but negative on ^{18}F-FDG PET/CT imaging should not be considered part of the GTV unless pathological data is available. Small nodes without uptake adjacent to the primary tumour or those located between pathological nodes, or with evident growth on consecutive CT scans, should be considered part of the GTV[89,90].

Disease confined to the chest

Postoperative RT: To date, postoperative RT (PORT) after surgery and sequential PCh of early -stage SCLC (N0) has not been shown to improve OS[91-93].

PORT can improve 5-year OS in pN2 disease[94-96], but does not significantly improve OS in stage pN1[75]. A MTA[97] concluded that PORT improves 1-3 year LC rates in pN1 and pN2 and 1-5 year OS in pN2 disease. However, PORT provides no benefit in pN0[98,99]. Clinical guidelines recommend PORT for the treatment of microscopic or macroscopic residual disease (R1 or R2) or pN2+ disease (level of evidence V, grade of recommendation C)[100,101].

The recommended treatment volumes are as follows: Bronchial stump, ipsilateral hilum, preoperatively involved regions, and pathologically-positive areas[102-104]. The treatment fields proposed in the randomised Lung ART trial for PORT in patients with pN2+ NSCLC[105] could be applied to patients with SCLC. However, PORT should be used cautiously in the treatment of SCLC given the negative results reported in a recent RCT in NSCLC[106]. Consequently, the treatment approach should be individualised and determined by consensus decision at the thoracic tumour board at each hospital.

The margin added to the clinical target volume (CTV) to define the planning target volume (PTV) will depend on the immobilization system used and other centre-specific factors. A 5 mm margin added to the involved LNs may be sufficient[107]. A 3 mm margin may be recommended for nodes < 2 cm and 12 mm for nodes > 2 cm[108].

Extracranial stereotactic radiation therapy

Respiratory motion should be compensated for by defining the internal target volume

(ITV) based on reconstruction of the phases of the respiratory cycle obtained through 4D-CT imaging[109]. Published series for SBRT do not differentiate between the GTV and CTV. The CTV margin for the PTV is 5 mm (range, 3-7 mm).

Radical RT

In the 1980s, studies were performed to determine whether delimitation of the GTV should be based on the pre- or post-induction PCh tumour volume[110], with the available evidence suggesting that there were no apparent differences in OS or relapse patterns. One study[111] evaluated 59 patients treated with neoadjuvant CT followed by CRT; 31 patients received TRT to the pre-PCh volume and 28 to the post-PCh volume. The use of the post-PCh volume did not increase out-of-field margin failures or thoracic recurrences. These data suggest that reduced fields are an acceptable strategy.

In a recent RCT[112] patients with stage I-III SCLC (92.9% stage III) underwent neoadjuvant PCh and were then randomised to TRT to the primary pre-PCh GTV (control arm) or the post-PCh GTV (experimental arm). Initially involved nodal regions were included in both treatments. There were no differences in locoregional PFS or OS at 3 and 5 years, although significant grade 3 esophagitis was observed in the pre-PCh arm. In the CONVERT and CALBG30610/RTOG 0538 trials, the treatment volume was the residual post-PCh GTV.

Another controversy surrounds the use of elective irradiation of mediastinal and supraclavicular LNs[27,28]. The study conducted by Baas *et al*[113] was the first to omit elective mediastinal irradiation. The volume irradiated was the involved primary tumour and LNs with a diameter ≥ 1 cm. In that study, 16% of patients developed local recurrence within the RT field. Updated data from that trial[114] reported out-of-field nodal recurrences in two patients and in-field recurrences in 9 (sole site of recurrence in 4). From the out-of-field relapses, one occurred in the ipsilateral supraclavicular fossae. The incidence of recurrences in the elective mediastinum is low (range, 2%-11%) and in more than 50% of cases these occur in the ipsilateral supraclavicular fossae, which underscores the crucial importance of accurate staging (with ^{18}F-FDG PET/CT and/or supraclavicular ultrasound)[115]. In the CONVERT trial, elective mediastinal irradiation was not permitted. In the CALBG 30610/RTOG 0538 (NCT00632853) trial, the CTV included the ipsilateral hilum. Staging with ^{18}F-FDG PET/CT was not mandatory in either trial.

The post-PCh primary GTV and pre-PCh nodal areas should be included in the treatment volume. For the primary GTV, if the tumour invades the mediastinum, the pre-PCh volume should be contoured at the tumour-parenchyma interface; only the residual post-PCh GTV should be considered. If CR in the primary tumour has been achieved, the primary CTV (CTVp) should be contoured on the pre-PCh image. Elective irradiation of areas located between involved mediastinal stations is optional, as is irradiation of the ipsilateral supraclavicular fossae in cases with extensive mediastinal involvement (level of evidence II, grade of recommendation B).

To account for microscopic disease after neoadjuvant CT, an adequate GTVp margin would be 1.4 mm. To reduce the irradiation volume, the CTV can be omitted with no significant differences in the recurrence rate. Micrometastases in the CTV may be small enough to be sterilized by PCh. In most studies, a 5-8 mm margin is added to the GTVp or ITV to establish the CTVp. When contouring the CTVn, the entire area encompassing the pathological nodes should be included with a 5-8 mm margin. The PTV margin is generally obtained by adding 5-10 mm to the CTV.

Metastatic disease

The optimal treatment volume for consolidation TRT in ES-SCLC has not been established[116-121]. Table 5 shows the volumes used in recent studies. According to ESTRO/ACROP recommendations, the following should be included: Post-CT GTVp, the hilar region, and the mediastinum (not only the involved area), in all cases accounting for the decreased nodal volume after Ch (R).

PCI

The indication for PCI in ES-SCLC is controversial[122]. Standard WBRT is recommended. To protect the hippocampus, this structure should be contoured according to the RTOG atlas[123] on a T1-weighted 3D contrast-enhanced MRI. For hippocampal protection, the planning CT should have a slice thickness ≤ 1.25-1.3 mm to ensure correct fusion with the MRI. In this case, the CTV should be defined as the whole brain minus the bilateral hippocampus + 5 mm[124,125]. To define the PTV, a 3-5 mm margin is added to the CTV to protect the outer table of the skull to avoid alopecia.

Table 5 Planning volumes in the principal studies of concurrent chemoradiotherapy in small cell lung cancer

Ref.	Study	Volume
Jeremic *et al*[41] 1999	Prospective, randomised	GTV + hilum + 2 cm; entire mediastinum + both supraclavicular fossae + 1 cm
Zhu *et al*[116] 2011	Retrospective	Primary GTV + GTVn > 1 cm short axis
Yee *et al*[117] 2012	Prospective, phase II	GTVp + GTVn visible on planning CT
Slotman *et al*[44] 2015	Phase III, randomised	GTVp post-CT + 15 mm + ipsilateral hilum + nodes involved pre-CT
Luan *et al*[119] 2015	Retrospective	CR post-CT: Primary GTV bed and GTVn involved pre-CT; SD post-CT: GTVp + GTVn; PD post-CT: New GTVp + GTVn + GTV
Qin *et al*[118] 2016	Retrospective	GTV: Thoracic, mediastinal, and supraclavicular fossae
Gore *et al*[47] 2017	Phase II, randomised	Post-CT volume including the primary tumour and nodal areas involved at diagnosis
Luo *et al*[120] 2017	Retrospective	Post-CT GTVp + pre-CT primary tumour bed + GTVn of nodes involved pre-CT
Zhang *et al*[121] 2017	Literature review	CR: Mediastinum initially involved; PR: Residual pulmonary lesions + initially involved lymph nodes

GTV: Gross tumour volume; GTVn: Lymph node gross tumour volume; GTVp: Primary gross tumour volume; CT: Computed tomography; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Organs at risk

A fundamental aspect of RT planning in SCLC is to delineate the organs-at-risk (OAR) with adequate dose limitations.

Lung: The dose limits on the dose/volume histogram (DVH) for the lung are based on both lungs minus the target volume (GTV)[126,127].

DVH dosimetric parameters in TRT [V5, V13, V30, V20 and mean lung dose (MLD)] may be associated with the risk of pneumonitis, mainly grade ≥ 3. The most commonly used parameters are the V20 and MLD. When V20 is limited to ≤ 30%-35% and MLD ≤ 20-23 Gy, the reported rate of radiation pneumonitis is ≤ 20%[128]. The incidence of grade 5 pneumonitis is extremely low and associated with dose fractions totalling > 2 Gy, elevated V20 values, and lower lobe tumours.

Based on RTOG 0813 and 0915 recommendations, the 2016 SABR guidelines 2016, and data from TG 101[126], the recommended dose limits for SBRT per fraction are as follows: One fraction: 7 Gy/1500 cc, 7.4 Gy/1000 cc; three fractions: 11.6 Gy/1500 cc, 12.4 Gy/1000 cc, V20 < 10%, V12.5 < 15%; four fractions: 11.6 Gy/1500 cc, 12.4 Gy/1000 cc, V20 ≤ 12%, MLD < 6 Gy; five fractions: 12.5 Gy/1500 cc, 13.5 Gy/1000 cc, V12.5 < 15%, V20 < 10%; and eight fractions: V12.5 < 15%, V20 < 10%.

Trachea-proximal bronchial tree: The trachea should be contoured in the mediastinal window, including all layers. The upper limit is the cricoid cartilage and the lower limit is the upper border of the proximal bronchial tree (2 cm above the carina). The bronchial tree includes 2 cm distal from the trachea, the carina, the right and left main bronchi to the lower-lobe bronchi. Contouring ends at the segmental bifurcation.

The RTOG recommends the following dose/volume parameters for the trachea and ipsilateral bronchus: 20.2 Gy (Dmax)/10.5 Gy (< 4 cm^3) in one-fraction; 30 Gy (Dmax)/15 Gy (< 4 cm^3) in three fractions; 34.8 Gy (Dmax)/15.6 Gy (< 4cm^3) in four fractions; 105% of the prescribed dose to the PTV/18 Gy (< 4 cm^3) in five fractions; and 44 Gy (Dmax) in eight fractions[129].

Esophagus: All layers of the esophagus should be contoured from the cricoid to the gastroesophageal junction. Several factors—concomitant boost; AHF-RT; concurrent CRT—may increase the risk of severe acute esophagitis (grade ≥ 3), which can occur in up to 15%-25% of cases[130]. The V60 is the most accurate predictor of grade 3 esophagitis[131]: V60 < 0.07% = 4%, V60 0.07%-17% = 10%, and V60 > 17% = 22%. Dmean < 34 Gy may be associated with ≥ grade 3 acute esophagitis in 5%-20% cases; V35 < 50%, V50 < 40%, and V70 < 20% may limit ≥ grade 2 esophagitis to less than 30%[132]. The maximum dose is a better predictor of severe late toxicity than Dmean. In SBRT treatments, the incidence of ≥ G3 esophagitis is low; studies of single-fraction RT suggest that D2.5 cm^3 to the esophagus < 14 Gy will maintain a rate less than 5% and a Dmax < 22 Gy[133]. Other studies with one or more fractions report dose/volume limits as follow: D5cc <14.5 Gy; D2cc 15-20 Gy; and Dmax < 19 Gy[134].

Spinal cord: The spinal cord is contoured in the mediastinal window from the cricoid to below L2. The structure should be contoured following the bony limits of the spinal canal. Another possibility is to delineate the visible spinal cord on the CT and apply a 2 mm margin for the DVH analysis to estimate all uncertainties. In tumours located close to this organ, or for SBRT treatments, fusion with MRI is useful[126,127] The use of a planning OAR volume margin around a critical organ such as the spinal cord is sufficient to avoid overdose (level of evidence IV, grade of recommendation C).

In normofractionated treatments (1.8-2 Gy/day) that include the cord circumference, myelopathy rates of 0.2%, 6%, and 50% have been described after 50 Gy, 60 Gy, and 69 Gy, respectively. The NCCN recommends Dmax ≤ 50 Gy. In SBRT for tumours in which only part of the spinal cord is irradiated, the risk of myelopathy is < 1% with Dmax 13 Gy/1 fraction or 20 Gy/3 fractions. RTOG 0915 establishes limits of 14 Gy in one fraction and 30 Gy in 5 fractions; a strict limit of 22 Gy in five fractions should also be considered[135]. Although the main variable associated with myelopathy is the maximum dose, the volume irradiated is also important. When the D0.1cc parameter is used, lower dose levels are established in each fraction.

Brachial plexus: The brachial plexus is divided into five nerve roots, including C5 at the exit through the neural hole, and C4-C5 to T1 as they exit through the T1-T2 foramen. To delineate the brachial plexus in the mediastinal window setting, it is preferable to use IV contrast to distinguish the nerves from the adjacent vessels. If no contrast is used, the subclavian and axillary arteries and veins should be used for reference. In the treatment of apical tumours with SBRT, CT-MRI fusion is recommended[126,127]. The upper limit is the exit point of the C5 root through the C4-C5 neural foramen, while the lower limit is the subclavian and axillary neurovascular bundle not including the vessels to identify the lateral border, and the second rib as the medial limit; external limit: Space between the anterior and middle scalene muscles from the vertebral body of C5 to its insertion at the first rib; internal limit: At the space at the conjunction of the lateral border of the spinal canal and the space or soft tissue between the scalene muscles at the vertebral body. To reduce the risk of radiation-induced plexopathy[136], the Dmedian should be ≤ 69 Gy with a Dmax at 2 cm^3 < 75 Gy. The risk of plexopathy is greater for brachial plexus doses > 26 Gy in 3-4 fractions, and for maximum doses > 35 Gy and V30 > 0.2 cm^3.

Heart: The entire pericardial sac should be contoured, including the atria and ventricles. The upper limit is below the aortic arch (aorto-pulmonary window) and the lower limit is the cardiac apex, excluding the pulmonary artery trunk, aorta, and superior vena cava[126]. The trade-off between protecting the heart and the likelihood of controlling the lung cancer must always be considered.

In normofractionated RT, the risk of toxicity for Dmean values < 10 Gy, 10-20 Gy, and ≥ 20 Gy is 4%, 7%, and 21%, respectively. V50 values ≤ 25% and Dmean ≤ 20 Gy are recommended and the risk of pericarditis increases with pericardial Dmean > 26 Gy and V30 > 46% according to QUANTEC. In SBRT, dose/volume limits[135] are as follows: Four fraction treatments: Dmax < 45 Gy, V40 ≤ 1 cm^3, and V20 ≤ 5 cm^3. RTOG 0618, 0813, and 0915 used Dmax values of 22 Gy/16 Gy < 15 cm for single fractions, Dmax 30 Gy/24 Gy < 15 cm^3 in 3 fractions, Dmax 34 Gy/< 15 cm^3 in 4 fractions, and 105% of the dose prescribed at PTV/32 Gy < 15 cm^3 in 5-fraction regimens[129].

Great vessels: The great vessels are contoured in SBRT, including the aorta and vena cava, from 10 cm above the PTV to 10 cm below it. Dose limits are Dmax 37 Gy/31 Gy < 10 cm^3 in single fraction treatments, Dmax 45 Gy/39 Gy < 10 cm^3 in three fractions, Dmax 49 Gy/43 Gy < 10 cm^3 in four fractions and 105% of the dose prescribed at PTV/47 Gy < 10 cm^3 in five fractions.

Chest wall: In peripheral tumours treated with SBRT, the chest well is considered an OAR. Each rib should be contoured at 5 cm from the PTV, excluding the intercostal space. Descriptive parameters are: Dmax 30 Gy/22 Gy < 1 cm^3 in a single fraction, Dmax 36.9 Gy/28.8 Gy/< 1 cm^3 in three fractions, and Dmax 40 Gy/32 Gy < 1 cm^3 in four fraction schemes.

PCI IN LIMITED AND ES DISEASE

The capacity of standard PCh agents (cisplatin-etoposide) to penetrate the blood-brain barrier is limited and 60% of patients will develop BM within two years of diagnosis, which explains the importance of PCI. The value of PCI is supported by the highest

quality scientific evidence. Aupérin *et al*[33] performed a MTA of seven clinical trials (mostly LS-SCLC), finding that PCI resulted in an absolute decrease of 25.3% in BM and a 5.4% improvement in 3-year OS.

A RCT conducted in Japan compared PCI to MRI-based observation in patients with ES-SCLC[122], finding that PCI did not provide any survival benefit, thus calling into question the role of PCI in these patients. Nevertheless, while PCI is considered standard in LS-SCLC, some patients—such as those older than age 75 or with high risk factors for neurotoxicity due to pre-existing disorders—may opt for close follow-up with MRI.

Prior to initiating PCI after completion of CRT, a brain MRI should be performed for re-evaluation. In patients with chest CR, MRI performed after CRT shows an incidence of BM ranging from 20%-32%[137,138].

Another MRI-based study found that the cumulative incidence (prior to PCI) of BM was 6.67% 4 mo after the initial treatment, rising to 12.2% at month 5 (1 mo later) until gradually reaching 21.82% at month 9[139]. These data indicate that PCI should be administered after completion of CRT. BM were detected in 10% of SCLC patients in the computed tomography era *vs* 24% in the MRI era. The emergence of MRI reduced the use of PCI from 42% of patients to only 13%[140].

Recent data from non-randomised studies suggest that PCI significantly benefits patients with LS disease, with better OS in patients re-evaluated with MRI[141].

Data from retrospective studies involving patients with LS-SCLC who achieve CR after CRT suggest that PCI does not improve OS, provided that MRI and SRS are available; however, those same data show that PCI reduces the incidence of BM[142]. SRS is an effective treatment for selected cases of SCLC with BM[143].

A MTA of seven randomised trials involving > 2000 patients found that PCI improved OS and significantly decreased the incidence of BM compared to observation[144,145]. However, it is important to note that those trials were highly heterogeneous in terms of the OS analyses due to the wide range of imaging tests utilized.

PCI is the standard treatment for most patients with LS-SLC who respond to radical CRT (level of evidence I, grade of recommendation A). Although the MTA by Aupérin *et al*[33] included only patients who achieved CR after CRT, the role of PCI can be extrapolated to patients with partial response based on current radiological tests. The NVALT-11/DLCRG-02 Study and SWOG S1827 phase III (MAVERICK) non-inferiority trial (NCT04155034) are currently being performed to determine whether active surveillance with MRI yields similar survival outcomes to MRI plus PCI[146].

The use of PCI in patients with ES-SCLC is controversial. The EORTC study[38] showed that PCI reduced the incidence of BM from 40% to 15%, prolonged median survival (from 5.4 mo to 6.7 mo) and improved OS rates (27% to 13%). However, prior to initiation of PCI, no imaging test was required in that study. In the study conducted by Takahashi *et al*[122] all patients underwent MRI prior to PCI. The cumulative incidence of BM in the two arms (PCI *vs* observation) was 48% and 69%, respectively. Despite the lower rate of BM with PCI, this did not improve OS (Table 6).

The PCI dose in ES-SCLC is 2500 cGy in 10 fractions (level of evidence I, grade of recommendation A) and 2000 cGy in 5 fractions[38]; however, the neurocognitive effects of the latter scheme have not been fully investigated.

Although two MTA and one pooled analysis have shown that PCI improves OS in patients with ES-SCLC[147-149], the result of another MTA called into question the value of PCI[150]. Consequently, the decision to use PCI or not should be discussed with the radiation oncologist. Patients with ES-SCLC who progress after PCh should not receive PCI.

PCI is associated with several acute toxicities, including alopecia, headache, fatigue, and, in rare instances, nausea and vomiting. Long-term sequelae such as severe memory loss, intellectual impairment, or even dementia and ataxia (both rare) have been reported. Chronic neurotoxicity (CNT)[151] may be associated with age, high total doses, dose fractions > 3 Gy, and concomitant PCh. In the prospective RTOG 0212 trial[152], age was the most significant factor for the development of CNT, with 83% of patients over age 60 developing CNT at 12 mo compared to only 56% of younger patients.

A recent systematic review assessed the risk factors for CNT after PCI[153], concluding that there is a lack of sufficient quality data to define these factors. However, disease-related factors (paraneoplastic syndromes, undiagnosed cerebral micrometastases, steroid use, *etc.*) are known to influence the development of CNT. Numerous factors have been shown to exacerbate the toxicity of cancer treatment, including treatment-related factors (CT or CT-induced anemia); smoking; excessive alcohol intake; comorbidities (*e.g.*, depression and anxiety); hypertension;

Table 6 Summary of the main studies of prophylactic cranial irradiation in small cell lung cancer

PCI in SCLC								
Ref.	**Stage (LS or ES)**	**Phase**	**Treatment**	**Survival**	***P* value**	**Incidence of BM**	***P* value**	**Level of evidence**
Arriagada *et al*[144] 2002	LS	III	PCI; no PCI	18% at 5 yr; 15% at 5 yr	0.06	20% at 5 yr; 37% at 5 yr	< 0.001	A I
Aupérin *et al*[33] 1999	LS	Meta-analysis	PCI; no PCI	20.7%; 15.3%	0.01	0.38; 0.57	0.001	A I
Warde *et al*[20] 1992	LS	Meta-analysis	PCI; no PCI	HR 0.82		HR 0.48		A I
Takahashi *et al*[122] 2017	ES	III	PCI; MRI + no PCI	13.6 mo; 11.6 mo		48%; 69%		A I
Rusthoven *et al*[81] 2020		III	WBRT; SRS			5.2 mo; 6.5 mo	0.003	A I
Yin *et al*[145] 2019		Meta-analysis	PCI; observation	HR 0.81	< 0.001	HR 0.45	< 0.001	A II
De Ruysscher *et al*[146] 2018		III	PCI; observation	24.2 mo; 21.9 mo	0.56	7%; 27.2%	0.001	A I
Slotman *et al*[38] 2007	ES	III	PCI; no PCI	6.7 mo; 5.4 mo		15% at 1 yr; 40% at 1 yr	< 0.001	A I
Le Péchoux *et al*[154] 2009	LS	III	Standard dose PCI; high dose PCI	42%; 37%	0.05	29% at 2 yr; 23% at 2 yr	0.18	A I
Ref.	**Stage (LS or ES)**	**Phase**	**Treatment**	**Median Survival**	***P* value**	**Incidence of neurological deficit**	***P* value**	**Level of evidence**
Yang *et al*[157] 2018	LS	Meta-analysis	PCI; no PCI	HR 0.52		RR 0.5		A I
Viani *et al*[148] 2012	LS-ES	Meta-analysis	PCI; no PCI	OR 0.73	0.01			
Ge *et al*[149] 2018	ES	Meta-analysis	PCI; no PCI	HR 0.57	< 0.001	RR 0.47	< 0.01	A I
Brown *et al*[164] 2020		III	HA + WBRT + Memantine; WBRT + Memantine			Learning 11.5%, memory 16.4%; learning 24.7%, memory 33.3%	0.049; 0.02	AI
van Meerbeeck *et al*[165] 2019		III	PCI; PCI + HA			HVLT-R 28%; 29%	> 0.05	A I
De Dios *et al*[167] 2019	LS-ES	III	PCI; PCI + HA			FCSRT 21.7%, 32.6%, and 18.5% at 3, 6 and 12 mo; FCSRT 5.1%, 7.3% and 3.8% at 3, 6 and 12 mo	< 0.05	AI

LS: Limited-stage; ES: Extensive-stage; PCI: Prophylactic cranial irradiation; SCLC: Small cell lung cancer; WBRT: Whole brain radiotherapy; SRS: Stereotactic radiosurgery; HR: Hazard ratio; RR: Relative risk; OR: Odds ratio; MRI: Magnetic resonance imaging; HA: Hippocampal avoidance; BM: Brain metastasis; HVLT-R: Hopkins Revised Verbal Learning Test; FCSRT: Free and cued selective reminding test.

hyperlipidemia; and diabetes caused by vascular damage and cerebral hypoperfusion.

A dose escalation study[154] found no significant reduction in the 2-year incidence of BM at doses > 25 Gy, but did find an increase in mortality. A neurocognitive analysis of data from RTOG 0212 (2500 *vs* 3600 cGy) showed a significant increase in CNT with higher doses[152].

In patients with stage I SCLC, the risk of BM is minimal and there does not appear to be any survival benefit associated with PCI in surgically-treated stage pT1-2N0M0 disease[155-157]. Consequently, follow-up in this patient population should be limited to brain MRI.

Few studies have evaluated the impact of PCI on survival outcomes in elderly patients. Eaton *et al*[158] evaluated PCI in 1926 patients, finding that this treatment improved OS in patients ≥ 75 years of age but not in those ≥ age 80. Other series suggest that patients older than 65-70 years of age—especially those with large tumours and/or women—are unlikely to achieve an OS benefit even after a good response to CRT[159,160]. One study suggested that patients with LS-SCLC and incomplete response to CRT may not benefit from PCI[161]. Active surveillance with brain MRI is preferable in elderly patients and in those with limited functional status, pre-existing neurocognitive disorders, or significant comorbidities.

Neurocognitive deterioration associated with PCI[162] is caused in part by irradiation of the hippocampus. For this reason, hippocampal sparing techniques have been evaluated, with promising results in metastatic patients treated with WBRT and hippocampal avoidance[163,164]. As a result, several multicenter RCTs are currently underway. NCT01780675 is a trial[165] performed to assess the safety of PCI and its impact on memory with or without hippocampal avoidance in patients with SCLC. That trial included 168 patients, finding that the rate of cognitive decline [Hopkins Revised Verbal Learning Test (HVLT-R)] at 4 and 8 mo did not significantly differ for PCI alone *vs* PCI with hippocampal avoidance (28% *vs* 29%). Moreover, the incidence of metastases did not significantly differ between the groups, and none of the patients developed BM in the hippocampus[166].

The Spanish PREMER trial (NCT02397733) reported contrary results. Among the 118 eligible patients, a significant decrease in memory was observed in the PCI group *vs* the hippocampal avoidance group, as follows: At 3 mo: 21.7% *vs* 5.1%; at 6 mo: 32.6 *vs* 7.3%; and 12 mo: 18.5 *vs* 3.8%). That trial used the Free and Cued Selective Reminding Test (FCSRT) to test neurocognitive function, but this test (FCSRT) was not used in the other randomised trial[167].

The study endpoints of the NRG-CC003 trial (NCT02635009) were 12-mo intracranial relapse and 6-mo impairment of delayed recall on the HVLT-R[168].

The latest NCCN guidelines recommend hippocampal avoidance and the use of memantine during and after PCI because this delayed the time to cognitive deterioration in patients who received WBRT for BM (RTOG 0614)[169].

For the radiation oncologist, the decision to recommend PCI or not is challenging, as it is not always clear which patients are likely to benefit and which can be offered MRI surveillance. In some cases, surveillance with MRI may be preferred, especially for patients who show a CR to the initial treatment[80]. Risk assessment should be individualized and decision-making should be shared with patients[100,101].

RT TECHNIQUES

Image-guided radiation therapy (level of evidence IV, grade of recommendation C)

Published evidence on the role of image-guided radiation therapy (IGRT) in SCLC is limited. One study involving a series of 132 patients with SCLC[170] found no significant differences in OS between patients treated with or without IGRT. No toxicity information was reported.

IMRT (level of evidence IV, grade of recommendation C)

There are no randomised studies comparing IMRT to 3D-RT in LS-SCLC. Data from retrospective studies[171] suggest that OS is comparable, although IMRT is associated with a significantly lower rate of percutaneous feeding tube placement due to esophagitis (5% *vs* 17%).

Volumetric modulated arc therapy (level of evidence IV, grade of recommendation C)

Comparative dosimetric studies between IMRT and volumetric modulated arc therapy (VMAT), including patients with SCLC[172], show that each technique has specific advantages depending on the tumour location. In peripheral tumours, the lung V5 (%) is lower with VMAT than IMRT while the lung V30 (%) is lower with IMRT. In central locations with mediastinal involvement, VMAT has a lower V20 (%) than IMRT.

Proton therapy (level of evidence III, grade of recommendation D)

The first prospective study of proton therapy with concomitant CT in LS-SCLC was published in 2017[173]. The median dose was 63.9 cobalt gray equivalents (range, 45-66.6) in 33-37 fractions administered once [*n* = 18 (60.0%)] or twice daily [*n* = 12 (40.0%)]. Compared to IMRT, proton RT achieved a statistically significant reduction in mean doses to the spinal cord, heart, and lung, but not in mean esophageal dose or V20. At a median follow-up of 14 mo, LC and OS survival rates were 85% and 72% at one year and 63% and 58% at two years, respectively. There was only one case each (3.3%) of grade ≥ 3 esophagitis and pneumonitis. Grade 2 pneumonitis and esophagitis occurred in 10% and 43% of patients, respectively.

SABR

SABR treatment options include 60 Gy delivered in 3 fractions, 48 Gy in 4 fractions, and 50 Gy in 5 fractions for peripheral lesions. Doses of 40-45 Gy in 15 daily fractions or 50-55 Gy in 20-25 daily fractions are potential alternative treatment schemes during periods such as the recent COVID-19 pandemic[101,174] (level of evidence III, grade of recommendation D).

Compensation for treatment interruption

At least five studies have been published on compensation for treatment interruptions in SCLC. Of these, four have shown that delays in completing RT can have deleterious effects on the following: (1) Significant loss of locoregional control and worse OS/PFS outcomes, especially in patients who do not receive PCI after TRT and in patients with a SER (start and end of RT) interval ≥ 30-31 d[175-177]; (2) Significant decrease in 5-year OS, especially in men, (> 6 times higher)[178]; (3) Delays > 7 d for hypofractionated (40 Gy in 15 fractions) and normofractionated (50 Gy in 25 fractions) schemes, as well as delays > 29-30 d in patients treated with accelerated regimens (Turrisi scheme) appear to be deleterious[175-177]; (4) The only study that does not show a deleterious effect (subanalysis of CALGB-9235, F-III) involved normofractionated CRT (50 Gy), with RT initiated in the 4th cycle of CT (possible effect due to accelerated repopulation at the start of "late" RT after CT[179]; and (5) No values have been reported for Tk (time from the start of RT at which accelerated repopulation begins) or K (estimated loss of biological efficacy in Gy per day of delay that would need to be added to compensate). Similarly, compensation techniques for the Turrisi scheme have not been described.

The potential loss of radiobiological effectiveness due to treatment interruption should be considered as a risk exposure to potentially avoidable threats. Thus, this would not be subject to the rules of evidence-based medicine, which refers to the evaluation of the benefits of therapeutic/diagnostic techniques[180]. However, the following recommendations can be made: (1) Avoid and/or compensate if the interruption is > 7 d in hypofractionated (40 Gy, 15 fractions) or normofractionated (50 Gy, 25 fractions) schemes. By extension, this rule should apply to normofractionated schemes > 60 Gy (perhaps even when the interruption is > 5-7 d); (2) Avoid/compensate if total treatment time > 29 days in patients treated with accelerated schemes (Turrisi); (3) Avoid/compensate for interruptions, especially in men; and (4) compensation methods: In the absence of published studies, the following recommendations should be considered as general guidelines. For normofractionated schemes, any compensation method would be, in principle, appropriate. However, each case should be evaluated individually; the absence of *Tk* and *K* values makes it difficult to perform radiobiological calculations in cases in which it is necessary. For this reason, it would be preferable to use double treatment sessions and/or to perform RT on holidays/Saturdays whenever possible. For hyperfractionated schemes, compensation is more challenging for the same reasons, especially given the nature of such schemes. In addition to considering treatment on holidays/Saturdays, a reasonable alternative would be to increase the number of sessions (add 1-2 more days of treatment, which would mean 32 to 34 fractions rather than 30), or to increase the dose per fraction for the remaining sessions (increased the dose from 1.5 to 1.6-1.8 Gy/fraction), or to combine both approaches. In all cases, it is essential to assess OAR tolerances. If it is not feasible to continue with AHF-RT, another alternative would be to switch to a hypofractionated scheme (≥ 2.67 Gy/d).

REIRRADIATION IN SCLC

Very few studies have been published on reirradiation (reRT) in lung cancer. As a result, most of the available data come from retrospective studies, which are highly

heterogeneous in terms of histology (both SCLC and NSCLC), disease stage, treatments received (time interval between RT treatments, doses, fractionations, techniques, use of PCh or not, *etc.*) and in the parameters used to assess treatment response[181].

Approximately 30% of patients with SCLC are diagnosed with LS disease; of these 30%, will progress locally[32]. LC is an important factor that may influence OS. Thoracic recurrence can produce symptoms that affect the patient's quality of life and may even require emergency treatment. The review that included the largest number of lung cancer patients (any histological type) to date was published in 2017[182]. That study reviewed data from 13 clinical trials involving 435 patients treated with 3D-RT or IMRT and another 10 trials (253 patients) of SBRT.

Below we review and discuss the published reports on reRT that provide specific data for SCLC. One of the limited recommendations from these studies is the need to assess the indication for reRT in SCLC independently from other histologies due to the unique behavior of SCLC[183]. Reirradiation is especially indicated to treat hemoptysis and vena cava syndrome, but it is less effective for dyspnea[184]. Hypofractionated schemes are recommended, with doses > 40 Gy (EQD2)[185]. If the patient is in poor general condition (PS > 3) or presents extrathoracic metastases, reRT should only be performed in selected symptomatic patients. In these cases, reRT *vs* supportive treatment should be assessed.

In terms of the recommended doses and fractionations for reRT, reported treatment schemes range from 25-37.5 Gy in 9-15 fractions for symptomatic patients or those with metastatic disease. In general, the reported benefits of reRT in terms of symptom palliation lasts only for approximately 1 mo, probably due, in part, to the short OS in these patients [media survival: 1.7 mo (1.0-4.0)]. The risk of chronic toxicity is greater if there are overlapping fields in central tumours. Cumulative doses of 90-150 Gy in central structures should be avoided. If less than 6 mo have elapsed between one RT treatment and reRT, the spinal cord dose should be < 50 Gy (EQD2)[184]. However, if more than 6 mo have passed, doses of 40-45 Gy have been used in 20-25 fractions, with a safe cumulative mean dose of 87.4 Gy.

The following factors are associated with better OS: KPS > 70%; asymptomatic patient without metastatic disease; reRT dose > 40 Gy (EQD2); cumulative dose > 90 Gy (EQD2)[185]. Predictors of good response are PS, small recurrence, and a long interval between RT.

Based on the available data, we can conclude that reRT for SCLC at palliative doses (< 40 Gy) is useful to treat symptoms such as hemoptysis, vena cava syndrome, and rib pain; however, this technique should only be used in selected patients. In asymptomatic patients without distant disease and good PS, higher doses may improve both quality of life and OS. Thus, we recommend treating selected asymptomatic patients without metastatic disease with radical intent RT; in other cases, we recommend considering hypofractionated reRT *vs* supportive treatment to prevent toxicity.

APPENDIX: LEVELS OF EVIDENCE

The present review was carried out by Spanish radiation oncologists from the GOECP/SEOR to review RT and combined treatment of SCLC. This review is based on a quality analysis of the published literature according to internationally-accepted classification of the levels of recommendation and the grades or quality of scientific evidence[188].

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OPINION REVIEW

Cardiovascular risk management in cancer survivors: Are we doing it right?

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Abstract

Although under-recognized, cancer survivors continue to be at an increased risk of death from cardiovascular complications post-remission or cure. This increased burden of cardiovascular disease results from the interplay of various factors. Adequate cardiovascular risk assessment and timely intervention through a multi-disciplinary approach in these patients plays a pivotal role in the prevention of cardiovascular morbidity and mortality. We discuss the shortcomings of using current risk prediction scores in cancer survivors and provide some insights into cardiovascular risk management relevant for primary care physicians, oncologists, and cardiologists alike.

Key Words: Cancer survivors; Cardio-oncology; Atherosclerotic cardiovascular disease risk estimator; Cardiovascular disease risk; Childhood cancer survivor study; Cancer policy

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Core Tip: Accurate assessment of cardiovascular risk in adult cancer survivors is important. Cancer survivors form a unique subset of a vulnerable population given their exposures to potential cardiotoxic therapies. The importance of common preventive cardiovascular strategies in the long-term outcomes of cancer survivors is understated in clinical practice.

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INTRODUCTION

A 40-year-old African American female, with a past medical history of Hodgkin's lymphoma, and hypertension, comes to your clinic for follow-up. At age 18, she was diagnosed with Hodgkin's lymphoma and treated with doxorubicin, bleomycin, vincristine, etoposide, prednisone, and cyclophosphamide chemotherapy along with 24Gy radiation to the neck, mediastinum, and para-aortic nodes. Today, her blood pressure is well controlled with losartan. She has never smoked, does not have diabetes, and has a normal body mass index. Her last lipid profile revealed total cholesterol of 197 mg/dL, high-density lipoprotein cholesterol of 36 mg/dL, and low-density lipoprotein cholesterol of 156 mg/dL.

There are mainly two questions discussed here: How would you calculate her cardiovascular disease (CVD) risk? How can you reduce the risk of future cardiovascular events in her case?

According to the commonly used American Heart Association/American College of Cardiology (AHA/ACC) atherosclerotic cardiovascular disease (ASCVD) risk estimator, her 10-year risk of cardiovascular disease is 3.8%, putting her into the low-risk criteria[1]. However, this does not account for the increased risk of cardiovascular events from her exposure to potentially cardiotoxic chemotherapy and radiation, both known to increase cardiovascular (CV) morbidity and mortality in cancer survivors[2,3]. Here, we discuss the shortcomings of using ASCVD risk calculators in cancer survivors and provide some insights into cardiovascular risk management among cancer survivors relevant for primary care physicians, oncologists, and cardiologists alike.

As we know, advances in cancer medicine have resulted in long-term remission and even cure in some early-stage cancers. Although less recognized, cancer survivors continue to be at an increased risk of death from cardiovascular complications post-remission or cure[4]. Interestingly, cardiovascular disease is now one of the leading causes of death among long-term breast cancer survivors[5]. This increased burden of the cardiovascular disease results from the interplay of various factors such as chemotherapy (type, formulation, or cumulative dosage), exposure to radiation therapy or indirect factors (stress, deconditioning from cancer), or other pre-existing patient-related risk factors (increased age, obesity, hypertension, and diabetes). Also, demographic and socioeconomic disparities in cancer survivors could have an additive effect on outcomes[6]. Thus, adequate cardiovascular risk assessment and timely intervention through a multi-disciplinary approach in these patients play a pivotal role in the prevention of cardiovascular morbidity and mortality in this patient population.

CARDIOVASCULAR RISK PREDICTION

For childhood cancer survivors, a risk prediction tool for heart failure, ischemic heart disease, and stroke (by age 50) currently exists[7]. The childhood cancer survivor study cardiovascular risk calculator includes parameters such as type and dose of chemotherapy, and dose and extent of radiation in addition to the conventional cardiovascular risk factors utilized by the ASCVD risk calculator (Table 1). Going back to our patient, the estimated probability of developing heart failure for her by 50 years of age is 13.7% (95%CI: 7.9%-19.5%) with a relative risk of 33 (95%CI: 18.0-60.7) for developing heart failure compared to a non-cancer sibling. This would put her at intermediate risk (7.5% to 19.9%) of future cardiovascular events. Thus, the ACC/AHA ASCVD risk calculator severely underestimates the risk of future cardiovascular events and its use is not sufficient in this special group of patients. A similar model was developed for predicting heart failure and cardiomyopathy in breast cancer patients, however, it was limited to trastuzumab exposure[8]. Currently, no validated risk prediction tools exist for accurate cardiovascular risk assessment in cancer survivors who have undergone cardiotoxic chemotherapy or radiation treatments for adult cancers. Thus, it is imperative for primary care providers to

Table 1 Comparison of the risk factors included in the American Heart Association/American College of Cardiology atherosclerotic cardiovascular disease risk calculator and the childhood cancer survivor study cardiovascular risk calculator

ASCVD risk calculator	CCSS cardiovascular risk calculator
Age	Age
Gender	Gender
Race	Age at diagnosis of cancer
Cholesterol-total cholesterol, HDL levels	Dyslipidemia medication use
Blood pressure-systolic, diastolic, medication use	Hypertension medication use
Diabetes	Diabetes Medication use
Smoking	Anthracycline chemotherapy exposure and dosage (if known)
	Alkylating chemotherapy exposure
	Platinum agents chemotherapy exposure
	Radiation exposure to the brain, neck, and chest
	Chest radiation dose
	Heart-specific radiation dose

ASCVD: Atherosclerotic cardiovascular disease; CCSS: Childhood cancer survivor study; HDL: High-density lipoprotein.

aggressively treat known risk factors in cancer survivors to reduce their cardiovascular disease burden.

Hypertension, hyperlipidemia, obesity, and diabetes are common comorbidities among cancer survivors, as with the general population[9]. Cancer therapeutics related cardiac dysfunction (CTRCD) [defined as a decline in ejection fraction (EF) from baseline > 10% to a value < 53%] is often diagnosed late as symptoms of heart failure appear. Anthracycline and trastuzumab are one of the most commonly used chemotherapeutic agents known to cause CTRCD. Anthracyclines lead to a dose-dependent irreversible structural damage to cardiomyocytes (Type I CTCRD) resulting in heart failure, whereas trastuzumab causes inhibition of signal transduction predominantly (reversible injury, type II) and has an additive effect to the cardiotoxicity from other modalities when used in a combinational anticancer treatment regimen[10]. Radiation-induced heart disease, from radiation treatment to prevent cancer recurrence is another cause for major cardiovascular events among long-term cancer survivors that need to be accounted for where assessing CVD risk among patients[11]. An area of active research is assessing the utility of various CV imaging techniques for the early detection of individuals at a higher risk of future CV events following cardiotoxic therapy. Myocardial strain assessment through echocardiography and cardiac magnetic resonance to detect myocardial fibrosis when other imaging techniques are nondiagnostic have been an important addition to the existing diagnostic tools to detect early myocardial injury and follow-up CTRCD[12]. However, early intervention and effective cardioprevention as guided by accurate risk assessment can delay the occurrence or possibly prevent CTRCD. Hence, aggressive risk factor reduction should be pursued in all cancer survivors as outlined below.

CARDIOVASCULAR RISK MANAGEMENT

Hypertension

As we know, patients with uncontrolled hypertension are at an increased risk of heart failure[13]. Given the increased incidence of cardiotoxicity of numerous anticancer agents, cardio-oncology experts recommend a target BP below 140/90 mmHg in general and below 130/80 mmHg in patients who have diabetes mellitus or kidney disease for the maximal benefit[14]. However, this should change with the new AHA/ACC hypertension guidelines[15]. A meta-analysis evaluating the efficacy of neurohormonal blockade in preventing the decline of left ventricular EF after chemotherapy suggested that patients on beta-blockers and/or angiotensin-converting enzyme inhibitors or angiotensin receptor blockers had higher EF at 4 wk follow up

compared to those on placebo with usual care[16]. Hence, these drug classes should be preferentially used as anti-hypertensives in patients with cancer for their added benefit in preventing cardiac dysfunction in cancer survivors.

Diabetes mellitus

Recent data suggest that diabetic risk reduction among cancer survivors was much lower compared to their non-cancer counterparts[17]. Uncontrolled diabetes, obesity, and hyperlipidemia have been shown to induce myocardial fibrosis which subsequently increases the incidence of symptomatic cancer treatment-related cardiotoxicity[18]. Hence, increased vigilance and multi-disciplinary collaborations between expert diabetic educator, a nutritionist and/or dietician, and primary care physicians are required for optimal diabetes management and maintaining the HbA1c goal of < 7% in most patients.

Obesity

Up to 40% of cancer survivors develop obesity placing them at an increased risk of adverse cardiovascular outcomes and secondary cancers in the future. Growing evidence suggests the importance of adequate weight management in improving overall health and wellbeing especially cardiovascular health among survivors. In a study of breast cancer patients treated with anthracyclines and trastuzumab, obesity was found to be an independent risk factor associated with greater cardiotoxicity when compared to non-obese patients[19]. Given the high prevalence of obesity and associated inactivity in cancer survivors, energy balance interventions need to be incorporated in the treatment plan right from the time of the first diagnosis. A motivating diet plan- and exercise-induced weight management have led to clinically meaningful weight loss and subsequent control of risk factors related to cardiovascular health[20].

Nutrition and exercise

The role of nutrition and exercise is often underestimated despite robust evidence on its effects on cardiovascular morbidity and mortality among cancer survivors. It has been reported that adult survivors of pediatric cancers were found to have low cardio-respiratory reserve compared to age-matched healthy individuals[21]. Exercise training has been shown to significantly improve the decline in cardio-respiratory reserve after cancer therapy[22]. A large-scale study called TITAN is currently underway to evaluate the effect of a multidisciplinary approach to reduce the CTRCD. Exercise training is the cornerstone of the intervention arm in the study along with pharmacy, nutritional counseling, and cardiology team support[23]. A specific model for exercise and physical training for cancer patients and survivors, called cardio-oncology rehabilitation has also been proposed to effectively manage the CVD risk factors in this vulnerable population[24].

While researchers continue to develop a tool to estimate the cardiovascular risk among adult cancer survivors, the physician should focus on managing these patients by utilizing the ABCDE steps to prevent heart disease which has been largely applied to breast and prostate cancer survivors but can be adapted for all types of cancer survivors[25,26]. A: Being Awareness of risks of heart disease and symptoms associated with cardiovascular illness, and Aspirin prophylaxis for eligible patients; B: Maintaining Blood pressure below cutoffs; C: Controlling Cholesterol levels and promoting Cigarette/tobacco smoking cessation; D: Diet and Diabetes management; and E: Exercise (Figure 1).

CONCLUSION

In conclusion, cardio-oncologist care must be initiated right from the pre-treatment stage for identifying high-risk patients and mitigating the factors known to contribute to cardiotoxicity to after treatment phase where future cardiovascular risk is frequently assessed and appropriate measures are undertaken to maintain long-term cardiovascular health. Recent progress in terms of utilizing advanced imaging techniques and identifying the role of cardioprotective medications to prevent the progression of ventricular dysfunction has been a step in the right direction, and now the urgent need is to direct future studies with the focus to develop an accurate cardiovascular risk prediction and assessment tool for adult cancer survivors to continue to provide comprehensive cancer. In the meantime, physicians should work collaboratively and must strive to optimize and aggressively manage cardiovascular

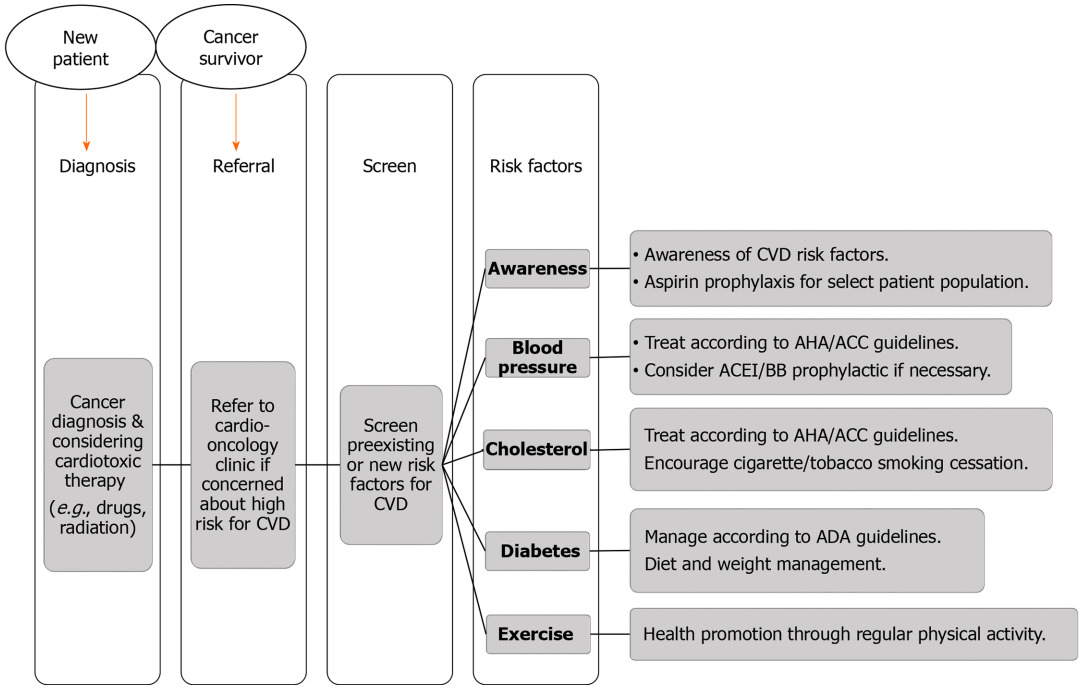


Figure 1 ABCDE steps to prevent heart disease in cancer survivors. CVD: Cardiovascular disease; AHA/ACC: American Heart Association/American College of Cardiology; ACE/BB: Angiotensin-converting enzyme/beta-blockers; ADA: American Diabetes Association.

risk factors in cancer survivors since they form a unique subset of a vulnerable population given their exposures to potential cardiotoxic therapies.

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MINIREVIEWS

Systemic adverse effects and toxicities associated with immunotherapy: A review

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Abstract

Immunotherapy is rapidly evolving secondary to the advent of newer immunotherapeutic agents and increasing approval of the current agents by the United States Food and Drug Administration to treat a wide spectrum of cancers. Immunotherapeutic agents have gained immense popularity due to their tumor-specific action. Immunotherapy is slowly transforming into a separate therapeutic entity, and the fifth pillar of management for cancers alongside surgery, radiotherapy, chemotherapy, and targeted therapy. However, like any therapeutic entity it has its own adverse effects. With the increasing use of immuno-therapeutic agents, it is vital for physicians to acquaint themselves with these

agreement of accountability for all aspects of the work.

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adverse effects. The aim of this review is to investigate the common systemic adverse effects and toxicities associated with the use of different classes of immunotherapeutic agents. We provide an overview of potential adverse effects and toxicities associated with different classes of immunotherapeutic agents organized by organ systems, as well as an extensive discussion of the current recommendations for treatment and clinical trial data. As we continue to see increasing usage of these agents in clinical practice, it is vital for physicians to familiarize themselves with these effects.

Key Words: Immunotherapy; Cancer; Malignancy; Adverse effects; Immune checkpoint inhibitor; Monoclonal antibody

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Core Tip: While immunotherapy is widely becoming a popular therapeutic treatment modality for a broad range of malignancies, it also has been known to cause a number of adverse effects. These effects have been found to be prevalent in a large number of organ systems. Clinicians should be aware of the various adverse effects associated with these novel therapeutic agents, as well as recommendations for management of these side effects in order to facilitate improved outcomes for patients undergoing these therapies.

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INTRODUCTION

Immunotherapy has become an increasingly popular therapeutic option for patients suffering from a variety of malignancies over the past several years. The development of these therapies began nearly two decades ago, when Leach *et al*[1] discovered that inhibition of cytotoxic T-lymphocyte associated protein 4 (CTLA-4) *in vivo* led to the rejection of tumor cells. Furthermore, in 2004, Chen described the role of programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) in the suppression of T-cell responses in the tumor microenvironment[2]. These discoveries have sparked extensive research in the field of immunotherapy, ultimately leading to the approval of ipilimumab (CTLA-4 inhibitor) by the United States Food and Drug Administration (FDA) for the treatment of melanoma in 2011[3]. Since then, immunotherapy has attracted immense attention as a therapeutic modality. The idea behind the utilization of these agents is based upon the premise of using the body's own immune system to attack and destroy cancerous cells[4]. Currently, immunotherapeutic agents have been approved for a wide variety of malignancies, including but not limited to melanoma, non-small cell lung cancer, colorectal cancer, bladder cancer, breast cancer, and head and neck cancers[5]. Although the success of these therapies has been well documented, the literature does report significant side effects closely associated with their use, known as immune related adverse effects (irAE). In this review, we provide a system-based overview of the documented adverse effects associated with the use of immunotherapy. It is essential for care providers to know these systemic adverse effects and toxicities when prescribing these agents for the treatment of cancers.

CLASSES OF IMMUNOTHERAPY AGENTS AND MECHANISMS

Immunotherapy works by stimulating the body's own immune system to target cancerous cells. With a better understanding of the workings of the immune system and the identification of new molecular targets, the classes of agents continue to grow.

Currently, immunotherapeutic agents can be classified into 5 major types including[6,7]: Immune checkpoint inhibitors (ICI); Adoptive cell therapy; Monoclonal antibodies; Treatment vaccines; Immune system modulators.

The National Institute of Health along with the approval of immunotherapeutic agents from the FDA have led to the recommendations for use of these agents in different types of cancers as summarized in Table 1. The mechanism of action of the 4 most frequently used immunotherapeutic agents is described as follows (see Table 2 for an overview of individual immunotherapeutic agents and their proposed mechanisms): (1) ICIs work through therapeutic targeting of the checkpoint molecules CTLA-4, PD-1, and PD-L1, which are normally utilized by healthy cells to promote self-tolerance and inhibit destruction by autoreactive T-cells[8]. However, tumor cells often utilize these proteins to avoid T-cell mediated destruction. By utilization of the ICIs, T-cells may have a greater ability to recognize tumor cells as foreign entities and promote cell lysis; (2) T-Cell transfer therapy is a treatment method in which a patient's T cells are collected and modified in a laboratory setting in order to improve their ability to bind and kill cancer cells. By collecting activated T-cells from cancer tissue, or genetically engineering T-cells, transfer therapy allows for passive immunization against cancers[9]; (3) Monoclonal antibodies are engineered to be antigen specific, often tumor-specific, and mediate the destruction of tumor cells *via* antibody-dependent cell-mediated cytotoxicity, promotion of phagocytosis or complement activation (referred to as complement-dependent cytotoxicity), or vascular and stromal ablation. A multitude of monoclonal antibodies have been developed to target various endogenous molecules[10]; and (4) Therapeutic cancer vaccines deliver concentrated antigen to both HLA class I and II molecules of antigen presenting cells, promoting both CD4 and CD8 T-cell responses which help to generate an anti-tumor response[11].

SYSTEM BASED CONSEQUENCES OF IMMUNOTHERAPEUTIC AGENTS

The following sections, organized by organ system, will describe and detail the potential adverse effects and toxicities associated with the use of currently approved immunotherapeutic agents. The systems specific adverse effects described are presented in the context of following systems: Cardiovascular, dermatologic, endocrine, gastrointestinal, neurologic, and pulmonary. In addition, we will provide an overview of some of the adverse events unique to chimeric antigen receptor (CAR) T-cell therapy.

CARDIOVASCULAR

The cardiovascular consequences of immunotherapeutic agents are being increasingly documented in medical literature, especially with the use of ICIs. The reported adverse effects can be further classified into two specific subtypes including structural, such as pericarditis and myocarditis, and arrhythmogenic, such as sick sinus syndrome. Cardiotoxicity has been most frequently reported with the use of ICIs and CAR T-cell therapy.

Structural consequences

The structural consequences may manifest in several ways. Patients may be asymptomatic, but laboratory investigations may report elevated markers of myocardial necrosis[12]. Furthermore, they can present with systolic dysfunction, symptoms of myocarditis, pericarditis, pericardial effusions, cardiac tamponade, or stress induced cardiomyopathies[12]. Structural cardiotoxicity secondary to the use of immunotherapy may be life-threatening and high suspicion for early recognition is imperative to prevent adverse outcomes.

The cause of cardiotoxicity due to immunotherapy is still largely under investigation. For immune checkpoint inhibitors, the presence of similar T-cell receptor sequences in tumor and in cardiac muscle is presumed to be the reason for myocardial and pericardial inflammation[13]. Furthermore, this can be confirmed with histopathological findings of fibrinous exudates with lymphocyte, plasma cell, and macrophage infiltration in patients with pericarditis[14]. Additionally, histopathological findings of endomyocardial CD8+ T-cell cells on post-mortem biopsy of those who had myocarditis secondary to immune checkpoint inhibitors also supports this

Table 1 Immunotherapy agents approved for treatment by the United States Food and Drug Administration in various types of cancers

Organ system	Cancer	Tumor marker	Staging at initiation of immunotherapy	Immunotherapy
Brain and central nervous system	GBM	IDH mutations, 1p19q deletion, MGMT promoter methylation, EGFRVIII amplification	Recurrent GBM	Bevacizumab
	Pediatric neuroblastoma	Homovanillic acid, Vanillylmandelic acid, LDH, NSE	High risk patients with partial response to first line agents	Dinutuximab
Head and neck	Head and neck SCC	CEA, SSC-Ag	Recurrent/metastatic SSC which progressed during/after platinum-based chemotherapy	Pembrolizumab
Breast	Breast cancer	BRCA1, BRCA2, CA 15-3, CA27.29, ER positive, PR positive, HER2/neu	HER2/neu positive	Trastuzumab
			Unresectable locally advanced or metastatic triple-negative, PD-L1-positive breast cancer	Atezolizumab
Gastrointestinal	SCC of the Esophagus	CEA, CA 19-9, SSC-Ag	Locally advanced or metastatic SSC, that progressed after treatment with one or more lines of standard therapy	Pembrolizumab
	Gastric and gastroesophageal junction cancer	CEA, CA 19-9, CA 72-4, DPD	Advanced cancer, that progressed despite two or more lines of standard treatment	Pembrolizumab
	Gastric cancer	CEA, CA 19-9, CA 72-4, DPD	Advanced cancer	Nivolumab (only approved in Japan)
	Pancreatic cancer	CA 19-9, DPD, CEA: Not frequently used	Advanced cancer with high microsatellite instability or high tumor mutational burden	Pembrolizumab
	Colorectal cancer	CEA, KRAS, BRAF V600, CA 19-9: Not frequently used, DPD, MSU, dMMR	Progressive CRC after Fluoropyrimidine Oxaliplatin and irinotecan treatment regimen	Pembrolizumab
Hematological	CLL	Beta-2-microglobulin, chromosome 17p deletion	Relapsed CLL, in combination with Fludrabine and cyclophosphamide	Ofatumumab
			Combination with Chlorambucil	Obinutuzumab
	B-cell precursor ALL	BCR-ABL	Relapsed/refractory Ph-negative B-cell Precursor ALL	Blinatumomab
	Follicular lymphoma	Beta-2-microglobulin	Relapse/refractory	Obinutuzumab
	Hodgkin's lymphoma	CD20	Non-responsive to therapy or relapse after > 3 therapies	Pembrolizumab
	Non-Hodgkin's lymphoma	CD20	Relapsed/progressing after autologous Hematopoietic stem cell transfer + Brentuximab Vedotrin or after 3 systemic therapies	Nivolumab
	Multiple myeloma	Beta-2-microglobulin, immunoglobulins	(1) Initial treatment in combination with dexamethasone, in patients eligible for autologous HSCT and maintenance therapy after autologous HSCT; (2) After > 3 therapies or non-responsive to proteasome inhibitor and immunomodulatory drug; (3) Combination with lenalidomide + dexamethasone or bortezomib + dexamethasone after > 1 therapy; And (4) Combination with revlimid + dexamethasone after 1-3 therapies	(1) Linalidomide; (2) Daratumab; (3) Daratumab; And (4) Elotuzumab
Respiratory	NSCLC	ALK gene, BRAF V600, KRAS, PD-L1, ROS1	Metastatic cancer expressing PD-L1 and progressing during/after platinum-based chemotherapy or with EGFR or ALK mutations	Pembrolizumab
			In combination with pemetrexed and carboplatin, with or without PD-L1 expression	Pembrolizumab
			Progressing during/after platinum-based chemotherapy	Nivolumab
			Progressing cancer while using approved therapy for the mutation in metastatic cancer expressing PD-L1 and progressing during/after platinum-based chemotherapy or with EGFR or ALK mutations	Atezolizumab
	Squamous NSCLC		First line in combination with gemcitabine + cisplatin	Necitumumab
Renal	Renal cell		Advanced RCC after antiangiogenic therapy	Nivolumab

	carcinoma			
Skin	Melanoma	BRAF V600	Unresectable cutaneous, subcutaneous or nodular lesions in relapsing melanoma after surgical resection	Talimogene Iaherparepvec (vaccine)
			Unresectable/metastatic	Pembrolizumab
			Unresectable/metastatic without BRAFV600 mutation	Nivolumab, Ipilimumab
			Adjuvant therapy for stage III	Ipilimumab
Urinary	Bladder cancer	Bladder tumor antigen, chromosome 3, 7, 17, 9p21 mutation, FGFR2, FGFR3 mutation	Locally advanced or metastatic bladder cancer, progressed during/after platinum-based chemotherapy or within 12 mo neoadjuvant/adjuvant treatment	Avelumab, Durvalumab, Pembrolizumab, Nivolumab, Atezolizumab
			Locally advanced or metastatic cancer with patient ineligible for platinum-based chemotherapy	Pembrolizumab, Atezolizumab

GBM: Glioblastoma multiforme; SCC: Squamous cell carcinoma; SSC-Ag: Squamous cell carcinoma antigen; CLL: Chronic lymphocytic leukemia; ALL: Acute lymphoblastic leukemia; HSCT: Hematopoietic stem cell transfer; NSCLC: Non small-cell lung carcinoma.

Table 2 Overview of some of the common immunotherapeutic agents and their proposed mechanisms

Agent	Mechanism of action
Ipilimumab	CTLA-4 inhibitor
Nivolumab	PD-1 inhibitor
Pembrolizumab	PD-1 inhibitor
Atezolizumab	PD-L1 inhibitor
Avelumab	PD-L1 inhibitor
Durvalumab	PD-L1 inhibitor
Talimogene Iaherparepvec	Cancer vaccine (directly destroys cancer cells, upregulates production of GM-CSF)
Necitumumab	EGFR inhibitor
Bevacizumab	VEGF inhibitor
Elotuzumab (anti-SLAMF7 monoclonal antibody)	Anti-SLAMF7 monoclonal antibody
Daratumumab (anti-CD38 monoclonal antibody)	Anti-CD38 monoclonal antibody
Lenalidomide	Immunomodulatory agent
Obinutuzumab	CD20 inhibitor
Ofatumumab	CD20 inhibitor
Blinatumomab	Bispecific T-cell engager
Trastuzumab	HER2/neu inhibitor
Dinutuximab	GD2-binding monoclonal antibody

CTLA-4: Cytotoxic T-lymphocyte associated protein 4; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1.

theory[15]. With respect to CAR T-Cell therapy, the mechanism of action of cardiotoxicity is presumed to be secondary to a cytokine storm generated by the reprogrammed T-cells leading to cardiovascular collapse[13]. Furthermore, like immune checkpoint inhibitors, common antigens between cardiac tissues and tumor cells, specifically the protein titin, has been hypothesized as a mechanism of action of cardiotoxicity from CAR T-cell therapy[13].

The literature also reports structural consequences with the use of monoclonal antibody therapy. Patient's with non-small-cell lung cancer treated with pembrolizumab, a PD-1 inhibitor, were found to have immune-mediated pericarditis[16]. Additionally, trastuzumab, a HER2 receptor blocker used in breast cancer treatment, is commonly known to cause cardiotoxicity. While the exact mechanism of cardiotoxicity

is unknown, studies have shown that the structural changes are different from those found with anthracyclines[17]. The most current hypotheses focuses on trastuzumab's interference in the integral HER2 signaling cascade resulting in the accumulation of toxic metabolites leading to cardiotoxicity. However, the exact molecular contributor is still unknown[18].

Treatment for the cardiotoxicity is centered around treating the over-active T-cell response with therapies such as high dose intravenous steroids, immunosuppressants, plasmapheresis, and intravenous immunoglobulins[14]. Furthermore, cessation of therapy should be considered with persistent cardiac symptoms longer than three months, if immunosuppressive therapy will need to be ongoing, or if there is evidence of life-threatening side-effects[14].

Rhythm consequences

Arrhythmias that have been well documented in the setting of immunotherapy use include atrial fibrillation, supraventricular arrhythmias, ventricular arrhythmias, and heart blocks[15].

The cause of rhythm consequences secondary to the use of immunotherapy is also still largely under investigation with even less knowledge about the mechanism when compared to structural consequences. If an arrhythmia presents in the setting of immunotherapy use, it is most often a result of persistent myocarditis that disrupts cardiac conduction[15]. However, post-mortem histopathologic analysis has demonstrated lymphocyte infiltration in both the sinoatrial node and the atrioventricular node, which provides strong evidence for a T-cell mediated insult similar to the mechanism of action of structural cardiotoxicity[15].

Patients who develop arrhythmias in the setting of immunotherapy should be treated with antiarrhythmics[15]. If the arrhythmia persists, an evaluation for a permanent pacemaker should be considered[15]. Similarly to structural consequences, cessation of immunotherapy should be considered with persistent cardiotoxic side effects and evidence of life-threatening consequences[14].

DERMATOLOGICAL

Dermatologic toxicities are extremely common with immunotherapy, most commonly resulting in a maculopapular rash and pruritis[19]. However, lichen dermatitis, psoriasis, bullous pemphigoid, as well as life-threatening adverse events like erythema multiforme or Stevens-Johnson syndrome (SJS) have also been reported[19].

The exact mechanism of causation of these lesions is not well understood, but several hypotheses have been put forth. Antibodies directed against melanoma-associated antigens can cross-react with the antigens produced by normal melanocytes. The presence of a Vitiligo-like lesion has been associated with higher objective tumor response rates.

Monoclonal antibodies are also frequently linked to dermatological adverse events. Rituximab, a CD20 antagonist, has been associated with morbilliform skin eruptions or even SJS, with the former requiring steroid therapy, while the latter requires immediate discontinuation of the agent[20]. Anakinra, an IL-1 antagonist, is associated with new-onset psoriasis as well as injection-site reactions. Tocilizumab, an IL-6 receptor antagonist, may rarely present with skin rash[20]. Ipilimumab, a CTLA-4 inhibitor used in the treatment of melanoma, has a diverse range of dermatologic adverse effects associated with its use such as rashes, folliculitis, mucositis, rosacea, SJS and vitiligo[20].

It is highly recommended for patients to undergo a dermatologic exam prior to starting immunotherapy to establish a baseline[19]. Depending on the severity of the condition, topical or systemic corticosteroids can be considered. However, prompt discontinuation is necessary in the event of severe reactions such as SJS[20]. Early detection and prompt management of these reactions is necessary as they profoundly diminish quality of life.

ENDOCRINE

Endocrine irAEs are also well documented, with the most common being acute hypophysitis and thyroid dysfunction[21]. Additionally, adrenal insufficiency, pancreatic dysfunction resulting in worsening glycemic control with subsequent

diabetic ketoacidosis (DKA) or hyperosmolar hyperglycemic state, and hypoparathyroidism have also been reported[21]. These adverse events are most commonly associated with ICI therapy. Conversely, oncolytic viruses, adoptive T-cell transfer, and cancer vaccines may rarely cause endocrinological dysfunction[22]. Currently, there are no specific recommendations for the screening of patients on ICI therapy for endocrine dysfunction, thereby making establishing a diagnosis of endocrine irAE quite challenging. Therefore, it is of critical importance that care providers monitor these patients closely to avoid morbidity and mortality secondary to the use of these therapeutic agents. The specific endocrine system related side effects, including proposed mechanism, presentation, and management strategies are discussed in detail in the following sections.

Thyroid

As discussed, thyroid dysfunction is one of the most common irAEs secondary to the use of ICI therapy. The mechanism of the development of thyroid dysfunction is currently being studied; however, there are several theories that have been proposed as the possible cause. First, it is assumed that increased immune system activation secondary to the use of ICIs may lead to an enhanced cytotoxic immune reaction that damages healthy tissue[23]. Second, it has been recognized that patients suffering from autoimmune damage associated with ICIs have an increase in the HLA-DR expression on CD14+CD16+ monocytes[23]. Some investigators have hypothesized that this overexpression may lead to excess monocytic activation, which infiltrate the thyroid gland, recognize self-antigens and induce subsequent cytotoxic damage to the normal cells[23]. However, it is not entirely clear whether the monocytes are directly responsible for the cytotoxic damage, or whether the damage is mediated by monocyte-activated autoreactive CD8+ T-cells. It has been well recognized that the incidence of autoimmune thyroid dysfunction occurs at much higher rate with PD-1/PD-L1 inhibitors as compared to CTLA-4 inhibitors. It is believed that this observation could be because healthy thyroid tissue has an increased expression of PD-L1, or because intra-thyroid lymphocytes have lower CTLA-4 expression[23].

Thyroid dysfunction could lead to features of hypo- or hyperthyroidism in these patients. Symptoms of hypothyroidism include fatigue, cold intolerance, weight gain, constipation, and hair loss, among many others. These patients may present with elevated Thyroid stimulating hormone (TSH) and low levels of T4. According to a retrospective cohort study by Ma *et al*[24], the median time to onset of hypothyroidism in patients receiving pembrolizumab (PD-1 inhibitor) was found to be 84 d (range: 43-544; P = 0.333) . For those receiving nivolumab (PD-1 inhibitor) and ipilimumab (CTLA-4 inhibitor) + nivolumab (PD-1 inhibitor), the median time to onset was 84 d (range: 14–154; P = 0.002) and 62 d (range: 21–141; P = 0.057) respectively[24].

Patients may also present with thyrotoxicosis, often secondary to thyroiditis or Graves' disease[21]. These patients will have typical features of hyperthyroidism such as weight loss, palpitations, tremors, diarrhea, and heat intolerance. Furthermore, many patients may commonly be asymptomatic, with the hyperthyroid state uncovered on routine laboratory investigations[21]. Ma *et al*[24] found that the median time to onset of thyrotoxicosis in patients treated with pembrolizumab (PD-1 inhibitor) was 44 d (range: 19-447; P = 0.593), while time for those receiving nivolumab (PD-1 inhibitor) was 56 d (range: 13-126; P = 0.004) and ipilimumab (CTLA-4 inhibitor) + nivolumab (PD-1 inhibitor) was 21 d (range: 7-64; P = 0.158). It is abundantly evident from these studies that the use of combination therapy leads to a more rapid symptom onset.

The treatment of thyroid dysfunction in these patients varies and depends on several factors such as whether the patient is suffering from hypo- *vs* hyperthyroidism, the severity of symptoms, and whether additional intervention such as hospitalization or surgery are indicated. The Society for Immunotherapy of Cancer has put forth guidelines for patients with hypothyroidism. These guidelines recommend that patients with severe symptoms with limitations of activities of daily living should discontinue ICI therapy, and should start standard thyroid replacement therapy[21]. Additionally, TSH and free T4 should be repeated after 6-8 wk of therapy to allow for titration of the dose. Resolution of symptoms is not needed prior to restarting ICI therapy, and these patients may resume ICI therapy once symptoms are no longer limiting self-care activities of daily living[21]. For patients with hyperthyroidism, the recommendation for discontinuation of ICIs is similar. In patients with severe symptoms that limit self-care activities of daily living, ICI should be discontinued immediately. Additionally, patients may benefit from beta-blockers for symptomatic improvement of hyperthyroid symptoms[21]. Of note, corticosteroids are not required for patients with thyroid irAEs.

Pancreas

Another frequently effected organ is the pancreas, as patients on ICIs have been reported to have diabetes as a consequence of the therapy[25]. These patients frequently present with symptoms consistent with DKA and may be subsequently diagnosed with new onset type 1 diabetes mellitus. However there have also been reports of insidious worsening of type 2 diabetes mellitus presenting as ketoacidosis as well[25,26]. The pathophysiology of the development of diabetes in these patients is not well understood, but it is postulated that it may be due to autoreactive CD8+ T cell activation against pancreatic beta cells as a consequence of the blockade of inhibitory pathways, such as the PD-1/PD-L1 pathway[27]. Patients may present with symptoms typical of hyperglycemia or ketoacidosis, including polyuria, polydipsia, weight loss, nausea, and vomiting. In a review of cases reported by Tan *et al*[25], it was found that the median onset of clinical presentation was 7.5 wk after starting ICI therapy, although cases have been described as early as one week or as late as one year after initiation. While more than two-thirds of the patients presented in DKA, only about 50% had positive auto-antibodies (anti-GAD, anti-insulin, anti-islet cell A, anti-zinc transport 8)[25].

Patients presenting with features of DKA should discontinue ICI therapy immediately and should be managed according to standard guidelines for DKA management[21]. If patients do not have DKA, but fasting glucose is in the 250-500 mg/dL range, ICI should also be held and the patient should be treated with insulin with a goal of fasting glucose level below 160 mg/dL[21]. All patients with new onset diabetes secondary to the use of ICIs should receive an endocrinology consultation.

Pituitary/adrenal insufficiency

Pituitary dysfunction and adrenal insufficiency have also been well documented irAEs of ICI therapy. In a meta-analysis conducted by Lu *et al*[28], the incidence of adrenal insufficiency was found to be 2.43% (95%CI: 1.73%-3.22%), while the rate of hypophysitis was reported to be 3.25% (95%CI: 2.15%-4.51%). Hypophysitis most frequently occurs secondary to CTLA-4 inhibitor therapy[28]. The specific mechanism is not well understood; however, multiple theories are currently being investigated. First, similar to all irAEs, it is believed that inhibition of self-tolerance leads to an autoimmune response against pituitary endocrine cells[29]. It has also been shown that CTLA-4 expression is greater on endocrine cells of the pituitary gland, especially prolactin and TSH secreting cells, which could possibly explain as to why both pituitary and thyroid dysfunction are the most common irAEs, and why symptoms occur more frequently in patients receiving CTLA-4 inhibitor therapy[29]. Patients may present with symptoms of headache, vision changes, and fatigue[21]. If patients subsequently develop adrenal insufficiency secondary to pituitary dysfunction, symptoms may include abdominal pain, vomiting, weakness, fatigue, hypotension, weight loss, and features of adrenal crisis. Additionally, the loss of function of other anterior pituitary hormones (FSH, LH, ACTH, TSH, Prolactin, GH) may lead to presenting symptoms consistent with the effects of the deficient hormone. Literature reports the median onset of clinical presentation to be 12 wk (range 3-76 wk) from initiation of ICI therapy.

Management of the pituitary dysfunction is focused on the replacement of the deficient hormone. In patients with adrenal insufficiency, steroids should be started to replace the corticosteroid deficit[21]. ICI therapy should be held in patients with moderate symptoms requiring local or non-invasive intervention, who have some limitation of instrumental activities of daily living[21]. ICI may be resumed when an initial work up is completed and the hormone replacement has begun[21].

GASTROINTESTINAL

The literature reports abundant gastrointestinal (GI) adverse effects associated with the use of immunotherapeutic agents. These have been reported across every class of immunotherapeutic agent, except for therapeutic cancer vaccines. In contrast to other cytotoxic therapies, cancer vaccines have reported minimal toxicities in a majority of clinical trials[30]. However these therapies are largely still in the development phase and adverse events will most likely be elucidated in the future.

The side effects associated with ICIs tend to be mild and transient with watery, non-bloody diarrhea being the most commonly reported[31]. Colitis, presenting with bloody diarrhea and fever, has also been reported. Typically, these adverse effects are more frequent with anti-CTLA-4 and anti-PD-1 agents[32]. Mouth ulcers, anal lesions like

fistulas, abscesses or fissures, and other extra-intestinal manifestations may also occur[33]. Some patients may also have asymptomatic elevation in the liver function tests[34]. Although the upper GI tract is rarely involved, some reported adverse effects include gastritis, esophagitis and aphthous ulcers[32].

The management of GI adverse effects as a result of ICI therapy is stratified based on the severity of the diarrhea or colitis. They are graded from 1-5 based on the severity, number of stools per day, and the need for hospitalization or additional intervention[35,36]. Treatment typically ranges from supportive (antidiarrheals, fluids/electrolytes) to hospitalization (IV corticosteroids and discontinuation of immunotherapy). Life threatening enterocolitis with perforation requires immediate surgical intervention.

T-cell therapy is associated with on-target adverse effects which are usually not life-threatening but may be treatment limiting. These on-target adverse effects are expected as the engineered T-cells may share specific target antigens with different organ systems with the most common T-cell adverse event being colitis[37]. The decision to discontinue T-cell therapy should be individualized to the patient and based on the severity of illness.

Monoclonal antibody agents have also been associated with diverse GI toxicities. Anti-angiogenic agents such as bevacizumab (VEGF inhibitor) and aflibercept (VEGF inhibitor) have been associated with dose-dependent GI perforations[38]. It is hypothesized that this may be due to ischemia secondary to limitation of blood flow to the GI tract[39]. The management of GI perforation includes prompt surgical intervention, bowel rest, and initiation of intravenous antibiotics. It has been reported that EGFR inhibitors such as cetuximab and panitumumab have a 66% increased risk of diarrhea compared to chemotherapy alone[40]. Hypomagnesemia is another common adverse effect of EGFR inhibitor therapy. It is dependent on the duration of the therapy and could possibly be the result of chronic diarrhea[41]. The management of diarrhea consists of bowel rest, hydration, electrolyte repletion, and the use of anti-motility agents. Hospitalization may be necessary in patients with severe dehydration. Finally, Trastuzumab (HER2/neu inhibitor) has been found to be associated with a higher prevalence of diarrhea compared to chemotherapy alone[42].

NEUROLOGICAL

Although rare, neurological irAEs are well reported side effects of ICI therapy, occurring in 1% of patients receiving monotherapy and 2%-3% of patients receiving a combination of ICIs[43]. Median onset of symptom occurrence is 6 wk; however, symptoms have been shown to occur as early as after the first dose and as late as at the 68th week after initiation of therapy[43]. Neurologic events are more commonly reported with CTLA-4 inhibitor therapy[44].

Numerous neurologic irAE have been reported. These include inflammatory myopathies, myasthenia gravis, vasculitis, small fiber sensory type neuropathies, immune-mediated neuropathies, cranial mononeuropathies, aseptic meningitis, autoimmune encephalitis, and multiple sclerosis (both exacerbation and new onset CNS demyelinating disease)[44]. The presentation is largely dependent on the type of irAE. The mechanism for neurological symptoms is most likely due to the increased T-cell and macrophage activation secondary to immune checkpoint inhibition, leading to autoimmune damage to parts of the nervous system[45]. Furthermore, it has been suggested that the aberrant T-cell activation and release of inflammatory cytokines may lead to sub-perineural edema and inflammation of the endoneurial microvessels, which is in part responsible for the nervous system dysfunction[46]. The treatment of the neurological adverse effects depends on the severity of the symptoms.

For any neurologic irAE, ICI therapy should be paused immediately, with considerations for possible permanent discontinuation of therapy if symptoms are severe (limiting ability to perform self-care activities of daily living) or progressive[21]. Moreover, patients with moderate symptoms or greater, based on limitation of daily activities, should be started on methylprednisolone (0.5 to 1.0 mg/kg/d for moderate; 1-2 mg/kg/d for severe) after a central nervous system infection has been ruled out as a cause of the neurologic dysfunction[21]. Additionally, in patients with involvement of the respiratory muscles, admission to the intensive care unit for close observation and ventilator therapy may be warranted[46]. The decision to restart the patient on immunotherapy should be a joint decision between the patient, oncologist and the neurology consultant.

Monoclonal antibodies and cancer vaccines have not demonstrated consistent

evidence of causing adverse neurological consequences. However, additional research is required to elucidate whether this is due to a lack of evidence or the true absence of adverse effects.

PULMONARY

The most frequently cited pulmonary adverse effects of immunotherapy are interstitial lung disease and concomitant pneumonitis. Ipilimumab, a CTLA-4 inhibitor, has been shown to be a common agent responsible for pulmonary adverse effects[47]. Most clinical trials reported a 3%-5% incidence of pneumonitis following treatment with immunotherapy. Studies by Delaunay *et al*[47] and Naidoo *et al*[48] reported that the incidence was higher in current or ex-smokers. A recent metanalysis by Jiang *et al*[49] investigated fatal immune-related adverse events by checkpoint inhibitors, with ten out of twelve trials evaluating CTLA-4 inhibitors. Based on the analysis, CTLA-4 inhibitors had a higher risk of fatal adverse events of pulmonary toxicity, with an overall risk of 20.5% (OR = 3.48, P = 0.10). A subgroup analysis was also performed according to the different dosing regimens and it was reported that the occurrence of toxicity with a 10 mg/kg dose was greater when compared to a 3 mg/kg dose (dose-dependent toxicity)[49]. The study by Nishino *et al*[50] reviewed ten different nivolumab (PD-1 inhibitor) trials alone or in combination with other immune checkpoint inhibitors for advanced cancers such as melanoma, lung cancer, and lymphoma, and identified pneumonitis in 11.8% patients. Radiological findings of sarcoid-like granulomatosis has also been reported in a patient receiving both anti-CTLA4 and anti-PD-1 agents. Dose-related adverse effects are less frequently encountered with the use of PD-1 inhibitors, and most toxicities are similar at doses ranging from 0.3 to 10 mg/kg.

The preferred initial modality for identifying features of pneumonitis is computed tomography scan. Pneumonitis can be classified as grade 1 with subclinical radiographic changes, grade 2 with mild dyspnea and cough, grade 3 and 4 with severe and life-threatening symptoms, and radiographic findings suggestive of acute interstitial pneumonia, acute respiratory distress syndrome, and/or cryptogenic organizing pneumonia[51]. The base of the lungs are most frequently involved, followed by the middle and upper regions; however, most often it may be mixed and multifocal. Bronchoalveolar lavage may be helpful in patients with cellular interstitial pneumonitis in that the fluid may show lymphocytosis, however there have been relatively few reports describing this finding and further research is needed[53].

The first step in management of all cases is the discontinuation of the immunotherapy, and initiation of corticosteroids[51]. The average duration of corticosteroid therapy also varies, but in most cases the median time is about 6.1 wk. Grade 1 Pneumonitis can be treated by interrupting immunotherapy, whereas grade 2 responds to oral corticosteroids. Grade 3 and 4 may need IV steroids, with some cases requiring infliximab, mycophenolate, tocilizumab, and IL-1 targeted agents[51].

Restarting immunotherapy in these patients is a challenge as pneumonitis can reoccur at any time; hence, close follow-up is recommended. Reinitiating ICIs can lead to recurrence of Pneumonitis in up to 33% of cases[52]. A flare of Pneumonitis can also occur during the steroid taper phase, and manifest similar to the initial episode. Data shows that steroids are responsive in subsequent flares as well as recurrence. Additionally, another challenging scenario is the relationship between radiotherapy and chemotherapy in aiding the development of side effects when used both prior to the initiation of immunotherapy as well as in combination with it[52].

TOXICITY RELATED TO CAR T-CELL THERAPY

CAR T-cell therapy is a type of T-cell transfer therapy currently approved for the treatment of several hematologic malignancies, including acute lymphoblastic leukemia and diffuse large B-cell lymphoma[53]. The mechanism of action involves the creation of CAR T-cells directed against the CD19 protein, which is present on B-cells[53]. While this treatment modality has proven effective in destroying chemotherapy-resistant tumor cells, it does not come without its own adverse events. It is thought that the irAEs resulting from CD19-directed CAR T cells are due to the fact that these modified T cells maintain their native receptors, and have the potential to expand rapidly once activated *via* interaction with the CD19 antigen[54]. These cells then have the potential to damage normal tissues expressing non CD19 antigens[54].

Several irAEs have been reported, including cytokine release syndrome (CRS), neurotoxicity, and hematologic abnormalities such as B cell aplasia, hypogammaglobulinemia, anemia, or thrombocytopenia[55].

CRS is an overwhelming release of cytokines, especially IL-1 and IL-6, that occurs as a result of CAR T-cell therapy. According to a review by Long *et al*[55], it may occur in up to 90% of patients. The symptoms of CRS vary greatly, and can involve a number of different organ systems. One of the most common symptoms is fever, and initial symptoms may mimic an acute infectious process[56]. In addition, there are several life-threatening complications that have been reported, including cardiac dysfunction, acute respiratory distress syndrome, neurologic toxicities, renal failure, hepatic failure, and hypercoagulability[55,56].

Laboratory findings in CRS are variable. Inflammatory markers such as C-reactive protein are often elevated[57]. In addition, patients may present with leukocytosis or leukopenia, anemia or thrombocytopenia, and elevated levels of D-dimer or ferritin[57]. Elevated levels of inflammatory cytokines, such as interferon gamma, IL-6, IL-10, or IL-2 may be present[57]. It should be noted that elevated levels of IL-6 are often found in CAR T-cell therapy induced CRS[57].

The National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE v5.0) has established a grading system to assess the severity of CRS[58]. The grading criteria for CRS according to the CTCAE v5.0 can be classified as follows[58]: Grade 1: Fever with or without constitutional symptoms; Grade 2: Hypotension responding to fluids; hypoxia responding to < 40% O_2; Grade 3: Hypotension managed with one pressor; hypoxia requiring ≥ 40% O_2; Grade 4: Life-threatening consequences; urgent intervention indicated; Grade 5: Death.

The management of CRS is centered around preventing life-threatening effects of the syndrome while allowing for the CAR T-cells to continue their anti-tumor activity[56]. For patients with grade 1 CRS, supportive care may be most appropriate treatment option[56]. Patients with grade 2 CRS may be treated conservatively, however for patients with extensive comorbidities and those of advanced age, treatment with Tocilizumab (IL-6 receptor inhibitor) and corticosteroids should be considered[56]. Finally, patients with grade 3 or 4 CRS should be admitted to the intensive care unit and treated with Tocilizumab (IL-6 receptor inhibitor) and corticosteroids. Tocilizumab has been evaluated and has been found to have considerable efficacy as a treatment option for CAR T-cell induced CRS[56,57]. Lee *et al*[56] recommend giving tocilizumab at a dose of 4 mg/kg over the course of one hour in adults. They also recommend repeating the dose if there are no signs of clinical improvement over 24 h to 48 h[56].

Neurotoxicity is another common adverse effect secondary to CAR T-cell therapy. According to a review by Gust *et al*[59], the incidence of neurotoxicity in patients undergoing CAR T-cell therapy varies between 2% to 70% of patients. The clinical syndrome of CAR T-cell induced neurotoxicity is often referred to as immune effector cell-associated neurotoxicity syndrome (ICANS)[59]. The most well known risk factors for developing ICANS are systemic cytokine release in addition to the severity of CRS[59]. Symptoms normally begin within the first 7 d following CAR T-cell infusion[59]. Symptoms include cognitive impairment, tremor, headache, altered levels of consciousness, seizures, and even coma[59]. The feared complication is cerebral edema, which is often fatal[59]. Currently, there is no evidence that blockade of a single inflammatory mediator is beneficial in ICANS. Therefore, it is recommended that corticosteroids be used as first line treatment due to their ability to reduce cerebral edema as well as to induce an immunosuppressive effect that decreases the secretion of pro-inflammatory cytokines[59].

CONCLUSION

Immunotherapy, a rapidly evolving therapeutic entity, has proven to be a worthwhile addition to the arsenal of therapeutic modalities care providers have against cancers. Advancement in the field is highly dependent on a better understanding of the immune system and the advent of newer agents. However, like any other therapeutic modality, it has its own set of adverse effects and toxicities. It is essential that physicians familiarize themselves with the adverse effects and toxicities associated with these agents as their role in management of cancers continues to rise. Additionally, proper patient education on the adverse effects of these agents along with the process of joint decision making between the provider and physician will hopefully result in a decrease in unfavorable outcomes. It is also worth noting that

immunotherapy is still in its infancy phase, and as newer, more specific therapies emerge secondary to a better understanding of the workings of the immune system, the ratio of benefits to risks may increase significantly.

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MINIREVIEWS

Overview of recent advances in metastatic triple negative breast cancer

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Abstract

Metastatic triple negative breast cancer (TNBC) has an aggressive phenotype with a predilection for visceral organs and brain. Best responses to chemotherapy are predominately in the first line. Recent studies have demonstrated improved progression free survival with the combination of atezolizumab/pembrolizumab and chemotherapy in programmed death-ligand 1 positive metastatic TNBC. However, a recent trial in a similar population showed no benefit for atezolizumab and paclitaxel which led to a Food and Drug Administration alert. Two phase III trials (OLYMPIAD and BROCADE3) demonstrated a benefit in progression free survival (PFS) but not overall survival in patients with BRCA-associated metastatic TNBC treated with Olaparib or Talazoparib respectively. For those treated with Talazoparib, the time to deterioration in health related-quality of life was also longer compared to chemotherapy. The BROCADE3 trial demonstrated that the combination of a platinum and veliparib increased PFS in first-line metastatic TNBC but at the cost of increased toxicity. There are no head-to-head comparisons of a poly (adenosine diphosphate-ribose) polymerase inhibitors (PARPi) and platinums. There are unanswered questions regarding the role of PARPi maintenance after platinum therapy as is standard of care in BRCA-associated ovarian cancer. Other areas of therapeutic interest include targeting aberrations in the phosphoinositide 3-kinase pathway, protein kinase B, mammalian target of rapamycin or utilising antibody drug conjugates. This review focusses on recent and emerging therapeutic options in metastatic TNBC. We searched PubMed, clinicaltrials.gov and recent international meetings from American Society of Clinical Oncology, San Antonio Breast Cancer Conference and the European Society of Medical Oncology.

Key Words: Triple negative breast cancer; Immunotherapy; Poly (adenosine diphosphate-ribose) polymerase inhibitors; Breast cancer

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Core Tip: Despite recent advances, chemotherapy remains integral to the management of advanced triple negative breast cancer. Immunotherapy and poly (adenosine diphosphate-ribose) polymerase inhibitors have shown much promise but have yet to demonstrate a proven overall survival benefit in this disease. Antibody drug conjugates and other targeted therapies may ultimately prove to be the next frontier in treating this illness.

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INTRODUCTION

Triple negative breast cancer (TNBC) accounts for approximately 15% of breast cancers and is characterised by the absence of expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 receptor (HER2) expression[1-3].

Triple negative breast cancers are most often high grade invasive ductal carcinomas which are characterised by an aggressive clinical phenotype. There are some rarer histological subtypes such as adenoid cystic carcinoma of the breast that is associated with an excellent prognosis.

Even for those with localised disease, approximately 25% of patients will relapse with distant metastasis. For patients with advanced or stage IV disease, the median overall survival (OS) is in the region of 12 mo with fewer than 20 % of patients alive at four-years. This is in stark contrast to ER-positive/PR-positive/HER2-negative (ER +/PR +/HER2-) disease where the median OS is closer to 36 mo and an estimated 40% of patients are alive at four years.

TNBC disproportionately effects younger women and black women, with these groups three-times as likely to be diagnosed with TNBC[4,5]. It has been estimated that 170000 women worldwide are diagnosed with TNBC each year of a total of 1 million breast cancer diagnoses[6]. It is also the most common breast cancer subtype in patients who carry a mutation in the *BRCA1* gene.

Advances in the treatment of HER2-positive breast cancer have resulted in clinical outcomes similar to those with ER+/PR+/HER2- disease however advances in triple negative breast cancer have been much slower[7]. In this article, we will review the biological features of advanced TNBC and explore the expanding treatment options for this aggressive disease.

CLINICAL FEATURES OF METASTATIC TNBC

Only 5% of patients with TNBC present with *de novo* metastatic disease[8]. The majority of patients unfortunately relapse following treatment with curative intent. The biological features of TNBC result in a unique clinical phenotype. It is characterized by a propensity for visceral and brain metastases, absence of bone metastases and typically early relapse (< 3 years).

Data from a Canadian breast cancer cohort with 180 TNBC (1601 in total cohort) patients showed that these patients were much more likely to develop distant recurrence (HR = 2.6, $P < 0.0001$) or death (HR 3.2, $P < 0.00001$) compared to other breast cancer subtypes. The risk of distant recurrence peaked at three years and declined rapidly thereafter[9]. A large cohort study from MD Anderson Cancer Centre identified similar patterns of distant recurrence and death[10].

TNBC is most commonly associated with visceral metastases including lung, liver and brain. Jin *et al*[11] identified 433 women with metastatic TNBC and found that 29% of them had 1 or greater brain metastases[11]. Median survival from time of diagnosis of brain metastases in this study was just 7.3 mo highlighting the significant mortality associated with intracranial disease.

THE BIOLOGY OF TNBC

Genomic features of TNBC

Triple negative breast cancer is characterised by the absence of expression of ER/PR/HER2. Almost 20 years ago breast cancer was classified using gene expression profiling into four main subtypes; Luminal A (ER+/PR+ with a low proliferation index), Luminal B (ER+/PR + with a high proliferation index), HER2-overexpressing (HER2+ disease) and basal-like. Although basal-like broadly corresponds to TNBC, the terms are not synomonous[1,3,12]. In one study, 70% of TNBC belonged to the basal subtype and 76% of basal-type tumours would be classified as TNBC[13]. A small proportion of basal-like tumours express ER or express HER2[14,15].

Importantly, basal-like tumours express cytokeratins such as CK5/6, cadherin as well as epidermal like growth factor (EGFR)[3]. Contrary to previous doctrine, it appears that basal-like tumours do not arise from normal breast tissue (basal cells) but instead arise from luminal progenitor cells[16,17].

Mutations in *BRCA1/BRCA2* are commonly associated with the basal-like subtype of breast cancer on a genomic level[18,19]. Of course, *BRCA1/BRCA2* is associated with a high lifetime incidence of all breast cancers[20]. However, the highest incidence of *BRCA1/BRCA2* is found within the triple negative subgroup. It is estimated that approximately 20% of patients with TNBC may harbour a germline defect in *BRCA1/BRCA2*[20]. As a consequence, it is now recommended that all patients with TNBC should have *BRCA1/BRCA2* testing particularly if they are under 50 years old[21]. It is hypothesised that *BRCA1/BRCA2* results in the suppression of basal-like genes thus a pathogenic mutation acts as an oncogene specifically within the basal subtype[20].

Conversely, basal-like breast cancers may be a surrogate for cancers which behave biologically similar to *BRCA1/BRCA2*-mutated disease. These cancers are considered under the term 'BRCAness'[20]. 'BRCAness' refers to cancers without *BRCA1/BRCA2* mutations but have other causes of homologous recombination deficiency (HRD) rendering susceptibility to poly adenosine diphosphate (ADP) ribose polymerase inhibitors (PARPi)[21]. Basal-like tumours associated with a *BRCAness* phenotype are characterized by high tumour grade, lymphocytic infiltrate, pushing margins, ER and HER2-negativity, an association with TP53 mutations, c-myc amplification, and multiple chromosome abnormalities[22]. Candidate genes which may result in a *BRCA-like* phenotype include *ATM, CDK1/2, PALB2* and many others. However, the clinical significance of these and their sensitivity to PARPi has generally been significantly less compared to patients with *BRCA1/BRCA2* mutations[23]. The American Society of Clinical Oncology (ASCO) Annual Meeting in June 2020 included presentations which showed objective responses similar to those seen in germline *BRCA*-mutation associated breast cancer in patients with somatic *BRCA* gene mutations and with *PALB2* mutations which are discussed later in this article.

Immunogenic potential of TNBC

The tumour microenvironment (TME) plays an important role in defining the interaction of our immune system with tumours. In TNBC, the TME is characterized by higher levels of vascular endothelial like growth factor (VEGF), tumour infiltrating lymphocytes (TILs) and tumour associated macrophages in contrast to other types of breast cancer[24]. Additionally, there is a high level of expression of TILs in patients with TNBC[24-29]. These have been shown to be a useful prognostic indicator across malignancies[30]. TNBC has been shown to have consistently elevated TILs in contrast to other subtypes and TILs have been shown to be associated with improved survival[29]. Ibrahim *et al*[29] found that patients with lymphocyte-predominant breast cancer had a 40% pathological complete response rate compared to 7% of those patients without[29]. High TILS are more frequent in TNBC (30%) compared to HER2-positive (19%) and luminal tumours (13%) and are associated with improved disease free survival and OS in early stage breast cancer[27,31,32]. This is consistent with findings in other malignancies demonstrating the important role of the immune system in cancer biology and prognostication. All of these features demonstrate that the TME of TNBC is highly immunogenic.

It is recognised that TNBC typically has higher levels of programmed cell death ligand [programmed death-ligand 1 (PD-L1)] expression in contrast to other subtypes of breast cancer[33-35]. PD-L1 has an important role in regulating our immune system, preventing overactivation of T cells and promoting the differentiation of regulatory T cells[36]. PD-L1 is the most agnostic and clinically utilised biomarker of response to checkpoint inhibition in patients with advanced malignancies. However, it's

sensitivity and specificity as an immunotherapy (IO) biomarker is variable across malignancies. There are several different antibodies used to detect it and there are also different staining algorithms adopted to measure it. This will be discussed in greater detail below (See PD-L1 assays).

TNBC has a relatively high tumour mutational burden (TMB) in contrast to other histological subtypes of breast cancer[37]. On average, TNBCs carry 1.68 somatic mutations per Mb of coding regions (approximately 60 somatic mutations in each tumour)[34]. The mutation burden is not uniform across TNBC, and some tumours have a high mutation burden (more than 4.68 somatic mutations per Mb) and a frequent occurrence of multiple copy-number aberrations involving genes that lead to multiple pathway alterations. TMB has been identified as a potential biomarker of IO response across malignancies[38]. There is a strong biological rationale for the use of TMB. Higher levels of TMB results in greater neoantigen expression and presentation to our immune cells enhancing our immune response. However, the clinical utility of TMB has not been fully demonstrated and it has failed to enter routine practice in most disease subtypes[39]. The Food and Drug Administration (FDA) has recently licensed pembrolizumab for the treatment of high TMB tumours (> 10 mutations/Megabase) with the FoundationCDx assay as a companion diagnostic[40].

THERAPIES IN METASTATIC TNBC

Chemotherapy

Chemotherapy remains the cornerstone of therapy in the treatment of metastatic TNBC (Table 1). It is well recognised that TNBC is intrinsically chemo-sensitive but unfortunately prone to rapid relapse and resistance, this is referred to as the triple negative paradox[41]. Most guidelines recommend a first-line anthracycline or taxane-based regimen for *BRCA1/BRCA2* wild-type patients who have not received these agents in the neoadjuvant or adjuvant settings[42,43]. There is evidence that patients may respond to re-challenge with these agents however most physician's would favour avoiding this in the case of anthracyclines due to the cumulative cardiac toxicity[44]. Much debate over the years has focused on the benefits of single-agent *vs* combination regimens. Combination regimens are now generally reserved for patients who are at-risk of or in visceral crisis[45]. Platinum-based regimens have demonstrated significant efficacy for patients with *BRCA1/BRCA2* mutant TNBC and other deficiencies in homologous recombination[46-48]. The TNT study directly studied platinum therapy responses in comparison to standard of care in advanced unselected TNBC[48]. The study, which randomised 376 patients to docetaxel *vs* carboplatin, found no evidence of a difference between carboplatin and docetaxel in objective response rate, progression free- or OS in the overall population. However, a prespecified subgroup analyses of patients with germline *BRCA1/BRCA2* mutations demonstrated improved Overall response rate (ORR) (68% *vs* 33%) and progression free survival (PFS) (6.8 mo *vs* 4.4 mo) but there was no OS advantage observed. The interpretation of OS is complex by the protocol specified planned cross over at progression.

Finally, a variety of other cytotoxic can be used in later lines of treatments including gemcitabine, capecitabine and the more recent addition-eribulin[49]. However, 30 years of experimentation with a variety of chemotherapeutics has yielded overall disappointing results. There is a significant unmet clinical need for newer more effective treatments which results in durable remissions for this patient population.

Targeted agents such as PARPi, drugs targeting the phosphoinositide 3-kinase (PI3K) pathway, immunotherapy and antibody drug conjugates are being incorporated alone or in combination with chemotherapy in treatment approaches.

IMMUNOTHERAPY IN METASTATIC TNBC

Monotherapy trials

In the Phase 1b KEYNOTE- 012 trial, published in 2016, patients with pre-treated TNBC were treated with pembrolizumab (Table 2) TNBC population as part of a larger basket trial[50]. A modest response rate of 18% (5/27) was seen with a further 25.9% of patients having stable disease. There was a suggestion of increased likelihood of response for patients with a higher PD-L1 score (P = 0.028).

In the JAVELIN Phase 1b trial, authors' investigated the use of avelumab in patients with metastatic, heavily pre-treated breast cancer with 58 patients in the group having

Table 1 Historical outcomes in metastatic triple negative breast cancer

	ORR (%)	PFS (mo)	OS (mo)
Single agent chemotherapy			
1L	10.0-28.0	3.5-5.4	9.9-17.5
2L	6.0-18.0	2.7-3.4	9.2-15.2
Combination chemotherapy			
1L	14.8-64.3	4.8-9.0	13.9-24.2
2L+	27.0[1]-60.0	2.9-7.0	8.1-16.5

[1]1-3Lines. Adapted from: Li *et al*[89]. ORR: Overall response rate; PFS: Progression free survival; OS: Overall survival.

Table 2 Immunotherapy as a monotherapy in metastatic triple negative breast cancer

Trial	*n*	Drug	ORR 1st line	ORR ≥ 1 prior line	Median OS (mo) 1st line	Median OS (mo) ≥ 1 line	Ref.
NCT01375842 Phase Ia	116	Atezolizumab	24%	6%	17.6	7.3	Emens *et al*[52], 2019
KEYNOTE-012 Phase Ib	32	Pembrolizumab		18.5		11.2	Nanda *et al*[50], 2016
JAVELIN/Phase Ib	58	Avelumab		5.2		9.2	Dirix *et al*[51], 2018
KEYNOTE-086 Phase II	170	Pembrolizumab	23.1%	5.3	18.0	9.0	Adams *et al*[53], 2019
KEYNOTE-119/Phase III	622	Pembrolizumab *vs* chemo		9.6 *vs* 10.6		CPS ≥ 1; 10.7 *vs* 10.2. CPS ≥10; 12.7 *vs* 11.6. CPS ≥ 20; 14.9 *vs* 12	Verret *et al*[83], 2019

ORR: Overall response rate; OS: Overall survival; CPS: Combined positive score.

TNBC[51]. The response rate within the TNBC cohort was disappointing at 5.2% with stable disease in a further 25.9% of patients. The combined positive score (CPS) was associated with higher likelihood of response (22.2% *vs* 2.2% within the TNBC population).

In a Phase 1a trial of atezolizumab in TNBC, authors' investigated the use of atezolizumab in TNBC in both the first line and second line setting[52]. Overall response rates were significantly higher in the first-line setting in contrast to the second-line setting (24% *vs* 6%) with a median duration of response of 21 mo. Patients with a higher immune cell (IC) PD-L1 score had improved clinical outcomes in contrast to patients with a negative PD-L1 IC.

In the KEYNOTE-086 study, authors' investigated pembrolizumab monotherapy in patients with heavily pretreated TNBC[53]. They included 170 patients in a single-arm phase 2 study. The majority of patients (61.8%) had PD-L1 positive tumours. Almost half of patients received 3 or more prior lines of therapy. Median PFS was modest at 2 mo with a 6 mo and 12 mo PFS of 14.9% and 6.2% respectively.

These early phase studies culminated in the phase III KEYNOTE-119 study which investigated pembrolizumab *vs* chemotherapy in patients who had received 1-2 prior lines of systemic therapy for patients with TNBC[54]. Patients had received at least one anthracycline or taxane based treatment and were randomised to either pembrolizumab or physician's choice of gemcitabine/eribulin/capecitabine. This study was powered for OS in the intention-to-treat (ITT) population. The PD-L1 immunohistochemistry (IHC) 22C3 pharmDX assay was used to determine the CPS on a specimen from a site of metastatic disease. Patients were randomised in a 1:1 manner between pembrolizumab and physician's choice of chemotherapy (n = 611). The majority of patients (61 %) had a CPS > 1. Pembrolizumab did not improve OS in patients with a CPS > 10 or CPS > 1 with a median OS of 9.6 mo for pembrolizumab and 10.6 mo for chemotherapy in the overall population. In an exploratory analysis, they did find that patients with a CPS > 20 had an improved OS with pembrolizumab (14.9 mo compared to 12.5 mo, HR 0.58). Grade 3-5 adverse events were significantly

higher in the chemotherapy group compared to the pembrolizumab arm (49% *vs* 34.9%). Although results only showed modest activity, it did suggest a relationship between efficacy and PD-L1 expression.

Combination studies-immunotherapy and chemotherapy

The early phase studies in metastatic TNBC indicated that treating patients with IO at earlier time points in their disease before exposure to multiple lines of treatment is associated with improved response (Table 2).

There was subsequently a shift of focus to combination chemotherapy and IO in TNBC (Table 3 and 4). In the phase 1a trial of atezolizumab and nab-paclitaxel, 33 patients were treated with the combination approach. The response rate was 39.1 % with a median duration of response of 9.1 mo. PD-L1 status did not stratify for responders. However, patients in the first-line setting had significantly higher response rates than those in the second-line setting or later (53.8% *vs* 30.0%)[55].

Phase III IMpassion 130 trial

This led to the pivotal IMpassion-130 study which was a phase 3, first-line study investigating atezolizumab + nab-paclitaxel *vs* nab-paclitaxel/placebo in 902 patients with advanced TNBC[56]. The trial was initially due to enroll 300 patients but the primary endpoint was expanded to include OS. The PD-L1 SP142 assay was used for PD-L1 assessment. Patients were excluded if they had completed treatment with curative intent < 12 mo before registration or if they had untreated or symptomatic brain metastases. The median PFS in the ITT population favoured the group receiving atezolizumab with a PFS of 7.2 mo *vs* 5.5 mo (HR = 0.80; 9, $P = 0.002$). However, within the PD-L1 positive subgroup (PD-L1 > 1%) the median PFS benefit was greater favouring the atezolizumab group with a PFS of 7.5 mo *vs* 5 mo (HR 0.62; $P < 0.001$). Final OS was presented at the European Society of Medical Oncology (ESMO) congress in 2020. In the ITT population, the median OS was 21 mo in the atezolizumab/nab-paclitaxel arm and 18.7 mo in the nab-paclitaxel arm (HR = 0.87; $P = 0.07$). The median OS in the PD-L1 positive group reached 25.4 mo in the atezolizumab arm *vs* 17.9 mo (HR 0.67; 95%CI: 0.53-0.86). However, this benefit was not statistically significant as the prespecified statistical hierarchical testing required a benefit to be seen in the ITT population to allow formal statistical analysis of the PD-L1 positive subgroup. No new safety signals emerged. Toxicity with combination approaches appears to be representative of the toxicity of each individual drug without evidence of synergistic effects thus far. The incidence of grade 3/grade 4 adverse events was higher in the atezolizumab arm (42% *vs* 32%). However, there was similar numbers of serious adverse events in each group (24% in the atezolizumab arm *vs* 19% in the placebo arm).

Phase III IMpassion 131

The IMpassion-131 study investigated if nab-paclitaxel could be replaced with paclitaxel in combination with atezolizumab in the first-line setting of advanced TNBC. Inclusion criteria were identical to the IMpassion130 trial, but the primary endpoint pertained to investigator-assessed PFS/OS tested first in the PD-L1 positive population. Patients were randomised in a 2:1 ratio to atezolizumab/paclitaxel *vs* placebo/paclitaxel ($n = 651$). In the PD-L1 positive population, there was no significant improvement in the atezolizumab arm with a PFS of 6 mo compared to 5.7 in the placebo arm (HR 0.82, 95%CI: 0.6-1.12). There were also no significant differences in PFS in the overall population (5.7 mo *vs* 5.6 mo). In an interim OS analysis, there was no significant differences in OS in the PD-L1 population (28.3 mo with placebo *vs* 22.1 mo with atezolizumab, HR 1.12, 95%CI: 0.76-1.65) or the ITT population (22.8 mo *vs* 19.2 mo, HR 1.11, 95%CI: 0.87-1.42). The trend towards an improvement in OS was somewhat of a concern for investigators and the medical oncology community. Further analysis demonstrated that patients in both arm had an equivalent exposure to paclitaxel. The reasons for this trend however remain unclear. Speculation includes the potential immune mitigating effects of dexamethasone usage for paclitaxel treatment. This trial resulted in an FDA alert warning against the use of paclitaxel in combination with atezolizumab in TNBC. No new safety signals emerged.

Pembrolizumab and eribulin were studied in a phase 1b study which enrolled 81 patients who had 0-2 Lines of previous treatment with advanced TNBC[57]. Overall response rate was disappointing-25.6%. Median PFS was again disappointing at 4.1 mo.

Another phase 1b study investigated (Table 3) the combination of pembrolizumab/capecitabine *vs* pembrolizumab/paclitaxel in the first-line setting in TNBC (*n*

Table 3 Early studies of Immunotherapy and chemotherapy in metastatic triple negative breast cancer

Trial	*n*	Drug	ORR 1st line	ORR ≥ 2 line	mOS 1st line	mOS 2nd line	Ref.
NCT01633970 Phase Ib	33	Atezolizumab + nab-paclitaxel	53.8%	30	24.2	12.4	Adams *et al*[55], 2019
KEYNOTE-150 Phase 1b/II	82	Pembrolizumab + Eribulin	25%	26.5	17.7	NE	Tolaney *et al*[57], 2018
Pilot and phase II. 1-2L[1]	29	Pembrolizumab + capecitabine or paclitaxel	43% pembro + cap. 23% pembro + paclitaxel		13.8 pembro + cap 7.9 pembro + pac		Page *et al*[90], 2019

[1]One to two lines of prior treatment. Pembro: Pembrolizumab; Cap: Capecitabine; Pac: Paclitaxel; ORR: Overall response rate; OS: Overall survival.

Table 4 Phase III first line metastatic immunotherapy + chemotherapy

	IMpassion130 (PD-L1 inhibitor)	Keynote-355 (PD1 inhibitor)	IMpassion131 (PD-L1 inhibitor)
Drugs	Atezolizumab/nab-paclitaxel *vs* placebo/nab-paclitaxel	Pembrolizumab + chemotherapy (nab-paclitaxel or paclitaxel or gemcitabine/carboplatin *vs* placebo + chemo	Atezolizumab/paclitaxel *vs* placebo/paclitaxel
ITT (*N*)	451 *vs* 451 (1:1 randomisation)	566 *vs* 281 (2:1 randomisation)	430 *vs* 221 (2:1 randomisation)
Inclusion	≥ 1 yr DFI	≥ 6 mo DFI	≥ 1 yr DFI
PD-L1 status	IC [positive (≥ 1%) *vs* negative (< 1%)]	CPS [positive (≥ 1%) *vs* negative (< 1%)]	IC [positive (≥ 1%) *vs* negative (< 1%)]
	SP142 antibody ventana platform	PD-L1 IHC 22C3 pharmDx kit	SP142 antibody ventana platform
Primary endpoints	PFS and OS in ITT population	PFS and OS by PD-L1 status (CPS ≥ 10 and ≥ 1) in ITT	PFS and OS in PD-L1 positive cohort
Median FU	18.0 mo (ASCO 2019)	25.9 mo and 26.3 mo (ASCO 2020)	8.6 and 9 mo (ESMO 2020)
PFS in PD-L1 +	7.5 mo *vs* 5 mo	9.6 mo *vs* 5.6 mo	5.7 mo *vs* 5.6 mo
OS in PD-L1 +	25.4 mo *vs* 17.9 mo	Awaited	22.1 mo *vs* 28.3 mo

PD-1: Programmed death 1; PD-L1: Programmed death-ligand 1; ITT: Intention-to-treat; DFI: Disease Free Interval; IHC: Immunohistochemistry; OS: Overall survival; ASCO: American Society of Clinical Oncology; ESMO: European Society of Medical Oncology; PFS: Progression free survival; CPS: Combined positive score.

= 28). Response rates were higher in the capecitabine cohort (ORR = 43%) compared to the paclitaxel cohort (ORR = 25%). Overall response rates were higher in those treated greater than > 12 mo from primary treatment (ORR 45% *vs* 27%).

Phase III KEYNOTE 355 trial

The much anticipated KEYNOTE-355 trial was presented at the inaugural virtual ASCO annual meeting in June 2020. This trial investigated pembrolizumab/chemo *vs* chemo (taxane *vs* gemcitabine/carboplatin) in patients with treatment-naïve, metastatic TNBC[58]. Patients were excluded if they had active brain metastases or recurrence of disease < 6 mo prior to primary treatment. PD-L1 was assessed with the IHC 22C3 pharmDx CPS assay in a central laboratory. The primary outcome measure was pre-defined as OS and PFS in the PD-L1 positive population (CPS > 1/CPS > 10) and the ITT population. In this trial, a hierarchial statistical testing method involved statistical testing of OS and PFS in the CPS > 10 group initially, followed by CPS > 1 and then the ITT population. The trial included 566 patients in the chemotherapy/IO arm *vs* 281 in the chemotherapy arm. In patients with a CPS score of 10 or greater, the median PFS favoured pembrolizumab with a PFS of 9.6 mo *vs* 5.6 mo (P = 0.0012, HR = 0.65). In patients with a CPS score of 1 or greater, the median PFS favoured the pembrolizumab arm with a PFS of 7.6 mo *vs* 5.6 mo (P = 0.0014, HR = 0.74). This was not statistically significant. This was similar to the ITT population where the PFS was 7.5 mo in the pembrolizumab arm and 5.6 mo in the placebo arm (HR = 0.82). OS data is awaited. This progression free survival improvement led to accelerated FDA approval for pembrolizumab in combination with chemotherapy in the first-line setting in November of 2020.

PD-L1 assays

A major challenge in IO trials is defining appropriate biomarkers to aid patient selection. However even within PD-L1, not all assays are equal[59]. The CPS utilises staining of both tumour and immune cells to reach a combined score which is thought to be enhance clinical utility of PD-L1[60]. Rugo *et al*[59] performed a post-hoc analysis of the IMpassion130 study investigating three PD-L1 assays; SP142, VENTANA SP263 IHC assay (IC ≥ 1%) and Dako 22C3^{A} assay (CPS ≥ 1, 22C3+)[59]. They found that the clinical benefit seen in patients with positive PD-L1 scores using the Dako 22C3^{A} and SP263 subgroups was driven by the SP142 PD-L1 subgroup. This study demonstrates that greater collaboration is needed to harmonise the assays utilised for PD-L1 scoring in clinical trials and clinical practice. The FDA appropriately has linked licensing approval of regimens with biomarker assays but this practice has not yet occurred in Europe.

In KEYNOTE-522, patients were randomised to receive chemotherapy + pembrolizumab *vs* chemotherapy + placebo[61]. Patients with PD-L1 positive and negative TNBC had an improvement in pathological complete response (pCR) with the addition of pembrolizumab. This is in contrast to the metastatic setting (in IMpassion130 and KEYNOTE-355), patients with high PD-L1 expression derived the benefit from the addition of IO. This would indicate that in the metastatic setting PD-L1 expression is required for response[56,58].

Adoptive immunotherapy approaches

Much of our focus in clinical practice involves utilising checkpoint inhibitors to enhance our immune response to malignancies. Adoptive immunotherapy involves infusing or adopting T cells or other immune cells in order to enhance the host *vs* malignancy response. Such approaches have been demonstrated to be effective in specific clinical circumstances. For example, tumor infiltrating lymphocytes have been used in melanoma and chimeric antigen receptor T cell therapies have demonstrated efficacy in leukaemia. There has been limited application of these treatments to TNBC thus far. Studies are limited to small numbers (< 10) of patients with limited evidence of activity. However, these treatments do offer a compelling rationale for harnessing the power of our immune system and it is likely they will be part of the treatment paradigm in years to come[62].

Take home message

Targeting PD-L1 in first-line, treatment naïve metastatic TNBC has resulted in the demonstration of clinical activity. The combination of atezolizumab and nab-paclitaxel has demonstrated an impressive 6 mo' OS advantage in the PD-L1 positive subgroup, however due to the hierarchial testing model, formal significance testing was not conducted. The phase III trial KEYNOTE-355 also demonstrated an improvement in PFS in patients with a CPS > 10 but OS data is awaited. The recently presented IMpassion-131 did not demonstrate any improvement in PFS and has led to an FDA alert cautioning against the use of this combination due to lack of efficacy and potentially increased toxicity. Further results will be needed to confirm the activity of IO in this setting.

It is important to note that all of these trials excluded patients that relapsed within either 6 or 12 mo of primary treatment. It is important that we do not extrapolate these clinical trial outcomes to our entire TNBC population.

Targeting homologous recombination deficiency in TNBC

PARPi offer a biologically appealing treatment for patients with intrinsic HRD. HRD renders cells vulnerable to neoplastic transformation. However, this vulnerability to neoplastic changes also renders tumour cells vulnerable to genotoxic cell death *via* PARP inhibition as cells are reliant on base excision repair by PARP so it represents an 'Achilles Heel'. By inhibiting two pathways of DNA repair, the tumour cells have impaired DNA replication. The combination of PARP inhibition and *BRCA1/BRCA2* mutations is termed synthetic lethality.

GERMLINE *BRCA1/BRCA2* MUTATIONS

Early stage clinical trials

In a proof of concept study published in the Lancet, authors' investigated olaparib in patients with advanced metastatic breast cancer (MBC) with germline *BRCA1/BRCA2*

(gBRCA) mutations. They investigated two doses of olaparib at 400 mg BD and 100 mg BD. Approximately half of patients in this study (26 of 51 patients) had TNBC with the remainder having other histological subtypes. Patients were heavily pretreated with a median of 3 prior chemotherapy regimens and platinum sensitivity was not needed for trial enrolment. Overall response rates were impressive in this heavily pre-treated population at 41% in the group receiving the higher dose and 22% in the group receiving the lower dose[63].

Kaufman *et al*[64] investigated olaparib further in a large phase 2 basket trial with 298 patients in a single-arm study[64]. Patients with any advanced solid-organ malignancy were included if they harboured a gBRCA mutation. In the breast cohort, patients may have received multiple lines of treatment and there was no requirement for platinum sensitivity. Response rates were modest with only 8 of 62 (12.9%) patients responding in this unselected population.

In the ABRAZO trial, investigators studied talazoparib in patients with MBC with g BRCA mutations in two cohorts (n = 84). In cohort 1, patients had responded to platinum based chemotherapy. In cohort 2, they had progressed through multiple lines of non-platinum based regimens and had gBRCA mutations. In cohort 1, 60% of patients had TNBC. Response rates in TNBC were modest at 26% (including both cohorts). There was a subset of patients with durable responses with 11% having prolonged response at the time of data cutoff[65].

In the phase II BROCADE trial, investigators studied the addition of veliparib in a randomised (1:1:1) trial with three arms with intermittent Veliparib/Carboplatin/Paclitaxel (VCP), Placebo/Carboplatin/Paclitaxel or Veliparib/Temozolomide[66]. Investigators identified a non-significant PFS benefit of 1.8 mo with the addition of veliparib to carboplatin/paclitaxel (14.1 mo *vs* 12.3 mo, HR = 0.79, P = 0.22) There was also no significant OS difference between these arms (28.3 mo *vs* 25.9 mo). The temozolomide/veliparib arm was significantly inferior with a median PFS of 7.4 mo and OS of 19.1 mo.

Phase III OLYMPIAD trial

In the phase 3 study, OLYMPIAD investigators (Table 5) studied olaparib in patients with MBC and gBRCA[67]. Half of patients had ER/PR-positive breast cancer with the remainder having TNBC. The cohort was heterogenous with 71.2% of patients having received any lines of treatments previously and 29.3% of patients having had prior exposure to platinum-based chemotherapy. Patients were randomised in a 2:1 manner (201:95) to receive olaparib *vs* standard therapy (capecitabine/eribulin/vinorelbine). Median PFS was significantly longer in the olaparib group in contrast to the chemotherapy group (7 mo *vs* 4.2 mo). In a subgroup analysis, the HR of benefit was significantly elevated in the TNBC group (0.43 *vs* 0.82 in the HR positive group). The response rate was 59.9% in the olaparib group *vs* 28.8% in the standard group. However, OS did not significantly differ between groups-19.3 mo in the olaparib group and 17.1 mo in the control group.

Phase III EMBRCA trial

In the pivotal phase 3 study EMBRCA, author's investigated talazoparib in 431 patients with gBRCA mutations and MBC[68]. Approximately half of patients had TNBC with the remainder having ER/PR-positive breast cancer. Patients had a median of 2 prior lines of chemotherapy and were randomised in a 2:1 manner to receive talazoparib *vs* physician's choice (eribulin/capecitabine/gemcitabine/vinorelbine). Median PFS was greater in the talazoparib group compared to the control group-8.6 mo *vs* 5.6 mo with an objective response rate of 62.6% *vs* 27.2%. Benefit within the TNBC and HR positive subgroups was equivalent. Crucially however, median OS was not significantly greater in the talazoparib group compared to the placebo group (19.3 mo *vs* 19.5 mo)[69]. Patients in the talazoparib group did however have improved health related quality of life outcomes. More than a quarter (25.5%) of patients suffered from a grade 3 or grade 4 adverse event in the talazoparib group which was similar to the control group (25.4%). Notably, one patient suffered from the rare but well described PARPi toxicity of acute myeloid leukaemia.

Phase III BROCADE3 trial

In the phase III study presented at ESMO in 2019, the BROCADE3 investigators compared VCP compared to carboplatin/paclitaxel in patients with MBC and a gBRCA mutation[70]. Patients were randomised in a 2:1 manner, with 337 patients in the veliparib group and 172 patients in the control group. Once again, half of patients had TNBC (52%). Only 19% of patients had previously received any line of treatment for

Table 5 Pivotal Phase III studies of poly adenosine diphosphate ribose polymerase inhibitors in patients with germline *BRCA1/BRCA2* mutations

Trial	*n*	Drug	Median PFS (mo) 1st line	Media PFS (mo) ≥ 1 line	Media OS (mo) 1st line	Media OS (mo) ≥ 1 line	Ref.
OLYMPIAD	296	Olaparib		7.3		19.3	Robson *et al*[67], 2017
EMBRCA	431	Talazoparib		8.6		19.6	Litton *et al*[69], 2020
BROCADE3 (1st line)	337	Veliparib	14.5		33.5		Bardia *et al*[77], 2020

PFS: Progression free survival; OS: Overall survival.

MBC. Patients had an improved PFS with veliparib compared to placebo (14.5 mo *vs* 12.6 mo, HR = 0.70). PFS in the ER/PR-positive group and TNBC groups were equivalent. However, OS was not significantly different between groups at an interim analysis (33.5 mo *vs* 28.2 mo, HR 0.95). The addition of veliparib did cause increased toxicities including any adverse event leading to discontinuation (15.6% *vs* 10.8%), anaemia (81.1% *vs* 69.1%), thrombocytopenia (79.6% *vs* 70.5%) and diarrhoea (48% *vs* 38.1%). At ASCO 2020, further analysis was presented which investigated patients who transitioned to monotherapy prior to progression in patients in either arm of the study[71]. In the VCP arm, 136 patients crossed over to veliparib monotherapy and 58 patients in the carboplatin/paclitaxel crossed over to monotherapy. The analysis suggests that the PFS benefit seen in the overall population is at least partially contributed to by those patients receiving veliparib monotherapy and the trial suggests significant antitumour activity with veliparib monotherapy. It remains unclear if a carboplatin induction regimen with PARPi maintenance may result in similar efficacy outcomes while sparing patients of some of the toxicity of combination approaches.

Beyond BRCA

The antitumor activity of PARP inhibitors has been established in *BRCA1/BRCA2* germline mutation carriers however whether they have a role in patients with somatic mutations in *BRCA1/BRCA2* or in germline mutations in DNA damage response genes other that *BRCA1/BRCA2* remains unclear. Recent studies have tried to provide data to answer the question.

In the TBCRC 048 study presented at ASCO in 2020, investigators studied the antitumour activity of olaparib in a basket study. Cohort 1 included patients with germline mutations in HRD excluding *BRCA1/BRCA2* and Cohort 2 included somatic mutations in these genes or *BRCA1/BRCA2*[72]. 27 patients were enrolled in cohort 1 and 26 patients in cohort 2. Most notably, only 19% of patients had TNBC with the majority of the remainder diagnosed with ER/PR-positive tumours. The most common mutations included *BRCA1* (6), *BRCA2* (9), *ATM* (10), *CHEK2* (8), *PALB2* (13). In the germline cohort, the overall response rate was 33% however all responses were in the *PALB2* cohort with an 82% response within that group. The median duration of response was 9 mo. For the somatic cohort, the overall response rate was 31% however all responses were in the *BRCA1/BRCA2* cohort with a 50% response within that group. The study met its primary endpoint of greater than 20% overall response rate in the cohort.

The SWOG S1416 study, presented at ASCO in 2020, investigated the combination of veliparib and cisplatin in patients with metastatic TNBC whom were mostly (70%) chemotherapy naïve[73]. Patients were enrolled and treated up-front with the combination approach. During their treatment, blood and tissue samples were analysed for g BRCA mutations, HRD score, germline non-*BRCA1/BRCA2* HRD associated mutations and *BRCA1* associated methylation mutations. The HRD score utilises loss of heterozygosity, telomeric allelic imbalance, and large-scale state transitions to develop a score which identifies tumours with a *BRCA-like* phenotype[74]. 37 patients with gBRCA mutations were identified, 101 patients with *BRCA1/BRCA2*-like phenotype (most identified *via* HRD score) and 110 non-*BRCA1/BRCA2* like patients. The gBRCA group was underpowered. Within the *BRCA1/BRCA2*-like group, PFS was significantly greater within the veliparib group in contrast to the placebo arm (5.7 mo *vs* 4.3 mo, P = 0.02). Within this same cohort, there was a numerical but non-

significant improvement in OS in patients in the veliparib group in contrast to the placebo group (13.7 mo *vs* 12.1 mo, $P = 0.14$). There was no improvement in PFS in the non-HRD group. No new safety signals emerged.

Take home message

In patients with gBRCA mutations, three phase III studies have demonstrated efficacy in terms of improvements in PFS and quality of life compared to chemotherapy. No study has demonstrated an OS advantage however cross over to a PARPi at progression complicates the analysis of this endpoint. These trials identified a subset of patients with long and durable responses however the majority of patients become resistant to these drugs (median PFS of 7 and 8.6 mo in the OLYMPIAD and EMBRCA study). Clinical trials in progress are examining PARPi in combination with immunotherapy and other combinations which may prevent the development of resistance to therapy.

Antibody drug conjugates

Antibody drug conjugates (ADC) offer the potential to deliver highly potent cytotoxic chemotherapy to tumour cells with reduced systemic toxicity (Table 6).

Sacituzumab govitecan-hziy

Sacituzumab govitecan (SG)-hziy is an ADC in which a topoisomerase I inhibitor, is coupled to the humanized antitrophoblast cell-surface antigen 2 (Trop-2) monoclonal antibody hRS7 IgG1κ through the cleavable CL2A linker. SN-38, a derivative of irinotecan, is subsequently delivered into the cells both intracellularly and into the tumour microenvironment and has demonstrated potent antitumour activity[75]. SG-hziy has been investigated in multiple epithelial tumours including TNBC. In a phase 2 single arm study, SG-Hziy demonstrated impressive response rates in a heavily pre-treated TNBC population[76]. 108 patients with metastatic TNBC were enrolled in the trial whom had multiple previous lines of treatment with a median of 3 prior treatments received. Overall response rate was 33% with 3 complete responses. The median duration of response was 7.7 mo with a median PFS of 5.5 mo. Notably, patients were able to remain on treatment longer than they had on prior therapies, suggesting a lack of cross resistance. The safety profile was acceptable with only 2.8% of patients discontinuing due to an adverse event. Grade 3 events included neutropenia (26%), anaemia (11%), fatigue and asthenia (11%). Grade 4 neutropenia was reported in 16% of patients.

At the ESMO congress in 2020, authors presented results from the ASCENT study, a randomized phase 3 study of sacituzumab govitecan ($n = 267$) *vs* treatment of physician's choice ($n = 262$) in patients (pts) with previously treated metastatic TNBC[77]. Patients had received at least 2 prior lines of treatment prior to enrolment. The primary outcome was investigator assessed PFS in the brain metastases free population. Progression free survival was significantly prolonged in the investigation arm with a PFS (5.6 mo *vs* 1.7 mo, HR 0.41, $P < 0.0001$). Median OS was significantly prolonged with SG (12.1 mo *vs* 6.7 mo, HR 0.48, $P < 0.0001$). The most common grade 3 or 4 adverse events with SG were diarrhoea (10%), anaemia (8%) and leukopenia (10%). Only 4.7% of patients discontinued the drug due to toxicity and there was no treatment related deaths.

Ladiratuzumab vedotin

Ladiratuzumab vedotin (LV) targets LIV-1, a transmembrane cell adhesion molecule highly overexpressed in TNBC. The drug's payload is the microtubule disrupting agent-monomethyl auristatin E. A Phase 1b/2 trial of LV in combination with pembrolizumab was investigated in a treatment naïve population with metastatic TNBC[78]. The trial was based on the biological rationale for a synergistic effect of the addition of two immune modulating agents in the first-line setting. 19 patients were included in the dose finding cohort with a further 32 in the dose expansion cohort. Patients were not pre-selected for LIV1 or PD-L1 expression. Response rates were encouraging at 54% in 26 evaluable patients regardless of their PD-L1 expression. Further work will be needed to clarify where LV may fit into the treatment paradigm for TNBC in the crowded field of the first-line setting.

Trastuzumab detuxtecan

Trastuzumab deruxtecan (DS-8201) is an ADC with an anti-HER2 antibody, a cleavable tetrapeptide-based linker, and a topoisomerase I inhibitor[79]. It has shown promising activity in HER2 + metastatic breast cancer and is part of the treatment

Table 6 Key phase I/II/III involving antibody drug conjugates and targeted therapies

Trial	*n*	Drug	ORR 1st line	ORR ≥ 2 line	mPFS ≥ 2 line	mOS 1st line	mOS ≥ 2 line	Ref.
NCT01631552 phase II	108	Sacituzumab govitecan		33%	5.5		12.4	Schmid *et al*[61], 2020
NCT03310957 phase I/II	51	Pembrolizumab + ladiratuzumab vedoitin	54%		-			Han *et al*[78], 2020
NCT029380341 phase Ib/II	21	US-1402		33%	-			Kim *et al*[82], 2019
NCT03279257 phase 1b/II	40	Alpelisib		57%	7			Sharma *et al*[84], 2018
LOTUS phase II	124	Ipatasertib		40%	6.2			Kim *et al*[86], 2017
NCT02978716 phase II	102	Trilaciclib	43%			20.6/17.6		Tan *et al*[88], 2019
ASCENT phase III	529	Sacituzumab Govitecan		35%	5.6		12.1	Bardia *et al*[77], 2020

ORR: Overall response rate; PFS: Progression free survival; OS: Overall survival.

paradigm post trastuzumab emtansine (TDM1) for HER2 + MBC[80]. However, there has been interest in the drug in patients with HER2 low tumours (IHC1 +/IHC2 + and FISH -) tumours. A phase 1b study investigated its utility in this subgroup with safety evaluated in 53 patients[81]. A total of 54 patients were included with a median of 7.5 treatments previously received. The objective response rates were encouraging at 37% and a median duration of response of 10.4 mo. However, the majority of patients had ER/PR-positive tumours with only 7 TNBC patients included. There was one of 7 patients who responded within the TNBC subgroup. Notably, 3 patients developed fatal drug induced interstitial lung disease.

US-1402

U3-1402 is a novel HER3-targeted antibody-drug conjugate designed with a peptide-based cleavable linker and a topoisomerase I inhibitor exatecan derivative (DXd) payload. It has a high drug-to-antibody ratio (approximately 8:1), and the stable linker is selectively cleaved by lysosomal enzymes upregulated in tumour cells[82]. It also exhibits bystander effect onto neighbouring tumour cells with antigen heterogeneity. A phase 1/2 multicentre, open label trial evaluated the safety and efficacy of the U3-1402 in HER2 negative, (including ER/PR-positive and TNBC) HER3 expressing advanced breast cancer. Among the 21 patients that received U3-1402, the ORR was 33% and disease-control rate (including complete response, partial response and stable disease) was 95%. Grade 3 or 4 toxicities included thrombocytopenia and increased liver enzymes[40].

TARGETED THERAPIES

Alpelisib

The PI3K pathway has been a focus of research in solid organ tumours due to its role in cell growth, deregulated apoptosis and association with both taxane and endocrine resistance[83]. Alpelisib is a potent, oral, class 1 inhibitor of the PI3K alpha isoform. A Phase I/II study investigated alpelisib plus nab-paclitaxel in HER2-negative MBC[84]. Patients were enrolled into the phase I dose expansion cohort (n = 10) or the efficacy phase II (n = 30) component. Among the cohort, 30% had TNBC and 74% of patients had received prior chemotherapy. Overall response rate was encouraging at 57% with a median PFS of 7 mo. However, within the PI3K mutated cohort, response rate was 65% with a median PFS of 13 mo. Results are encouraging that targeting the PI3K pathway may have clinical utility in TNBC.

Ipatasertib

The protein kinase B (AKT) pathway is commonly mutated in solid organ tumours playing a crucial role in cell survival and growth. AKT activation commonly occurs

through phosphate and tensin homolog (PTEN) loss or PIK3CA mutations. However, targeting the AKT pathway has proven to be challenging due to the associated toxicities. Ipatasertib is a potent AKT pathway signalling inhibitor which has demonstrated tolerability and antitumour activity in early clinical studies[85]. The LOTUS trial investigated ipatasertib in 124 patients in a randomised phase 2 study of ipatasertib/paclitaxel *vs* placebo/paclitaxel as first-line therapy for TNBC[86]. In the overall population, the median PFS was enhanced with ipatasertib (6.2 mo *vs* 4.9 mo, HR =0.6, P = 0.037). In patients with PTEN-low tumours (identified *via* immunohistochemistry), median PFS was 6.2 mo with ipatasertib *vs* 3.7 mo with placebo. However, within the PIK3CA/AKT1/PTEN-altered tumours, PFS was 9 mo *vs* 4.9 mo (HR 0.44, P = 0.041). The most common toxicity was diarrhoea in 23 % of patients in the ipatasertib arm leading to discontinuation in 3% of patients.

Trilaciclib

Trilaciclib is a potent, intravenous cyclin dependent kinase 4/6 (CDK4/6) inhibitor which is thought to acutely protect from cytotoxic associated myelosuppression and may promote immunogenic tumour cell death[87]. A phase II study of trilaciclib in TNBC in combination with the doublet of gemcitabine/carboplatin was designed to identify a reduction in myelosuppression associated with chemotherapy[88]. Patients (n = 102) were assigned in a 1:1:1 fashion to (Cohort 1) gemcitabine/carboplatin alone *vs* (Cohort 2) gemcitabine/carboplatin/trilaciclib (D1/D8) *vs* (Cohort 3) gemcitabine/carboplatin (D2/D9) and Trilaciclib (D1/2/8/9). Approximately 2/3^{rds} of patients were treatment naïve (in the metastatic setting). There was no significant difference in myelosuppression between the groups, however there was a significant OS benefit in the trilaciclib arms. Patients in Cohort 1 had a median OS of 12.6 mo *vs* 20.6 mo in Cohort 2 and 17.6 mo in Cohort 3.

CONCLUSION

Despite recent advances, metastatic TNBC remains an aggressive disease which predominantly affects younger patients. Recent advances in pre-clinical science have demonstrated an impressive rationale for the use of IO and PARPi.

There is evidence of activity for the use PD-L1 or PD-1 inhibitors in the first-line setting of TNBC. However, this has not yet resulted in statistically significant improvements in OS. Additionally, it remains unclear why findings with the combination of nab-paclitaxel and atezolizumab were not reproducible when atezolizumab was combined with paclitaxel. OS analysis from the KEYNOTE-355 study may assist us in reaching final conclusions for the up-front combination of IO and chemotherapy. However, these conflicting results suggest that the addition of IO into routine practice should be done so with caution.

Patients with g BRCA mutations have a consistent but modest PFS benefit of 1-3 mo across multiple phase I/II/III studies. However, these have unfortunately not translated into an OS benefit. While PARPi may have a future role in the treatment paradigm for TNBC, the OS benefits for patients remains unclear.

Encouragingly, antibody-drug conjugates and targeted therapies have demonstrated impressive response rates and PFS benefits in the monotherapy or combination settings in patients with TNBC. Most recently, SG has demonstrated an impressive 6 mo' OS benefit in a heavily pre-treated population. It is likely that SG will have a significant role to play in the future of TNBC in the monotherapy or combi-nation setting.

It is likely that the future of metastatic TNBC will involve treatment algorithms with combination approaches using chemotherapy, immunotherapy, PARPi, ADC and targeted therapies. Hopefully, the combination of the old and new will ensure that clinical outcomes continue to improve for our patients.

Clinical practice points

(1) Despite recent drug developments, chemotherapy remains integral to the management of advanced TNBC; (2) Immunotherapy and PARPi have shown much promise but have yet to demonstrate a proven OS benefit in this disease; and (3) Antibody drug conjugates and other targeted therapies may ultimately prove to be the next frontier in treating this illness.

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ORIGINAL ARTICLE

Observational Study

Thromboembolic events in metastatic testicular cancer treated with cisplatin-based chemotherapy

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Abstract

BACKGROUND

Testicular germ cell tumor (TGCT) is the most curable solid tumor and most common cancer among men 18-39 years. While cisplatin-based chemotherapy has significantly lengthened the survival of patients with TGCT, it is associated with a high rate of thromboembolic events (TEE).

AIM

To summarize our single-center experience highlighting patients who were diagnosed with TGCT and received platinum-based chemotherapy, with special attention to those patients who suffered a TEE.

METHODS

A retrospective analysis of the medical records and imaging studies of 68 consecutive individuals who were diagnosed with TGCT and received platinum-based chemotherapy at our Institution in a metropolitan community between January 1, 2014 and December 31, 2019.

RESULTS

A total of 19 (28%) patients experienced a TEE following orchiectomy which occurred during chemotherapy in 13 (68%) of these patients. Patients with a higher pathologic stage (stage III) were significantly (P = 0.023) more likely to experience a TEE compared to patients who had a lower stage. Additionally, patients who were treated with 3 cycles of bleomycine, etoposide, and cisplatin and 1 cycle of etoposide and cisplatin or 4 cycles of etoposide and cisplatin were

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significantly 5 (P = 0.02) times more likely to experience a TEE compared to patients who were treated with only 3 cycles of bleomycine, etoposide, and cisplatin.

CONCLUSION

Due to numerous factors that predispose to a TEE such as large retroperitoneal disease, higher clinical stage, greater number of chemotherapy cycle, central venous catheter, cigarette smoking, and possible cannabis use, high-risk ambulatory patients with TGCT treated with cisplatin-based chemotherapy may benefit from prophylactic anticoagulation. Randomized studies to evaluate the safety and efficacy of prophylactic anticoagulants are warranted in this young patient population generally devoid of medical co-morbidities.

Key Words: Oncology; Testicular cancer; Thromboembolic; Cisplatin; Pulmonary embolism; Thromboprophylaxis

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Core Tip: While cisplatin-based chemotherapy has significantly lengthened the survival of patients with testicular germ cell tumor, it is associated with a high rate of thromboembolic events (TEE). We analyzed 68 patients who were diagnosed with testicular germ cell tumor and received platinum-based chemotherapy. A total of 19 (28%) patients experienced a TEE following orchiectomy which occurred during chemotherapy in 13 (68%) of these patients. Patients with a higher pathologic stage (stage III) were significantly more likely to experience a TEE compared to patients with a lower stage. Randomized studies to evaluate the safety and efficacy of prophylactic anticoagulants are necessary in this young patient population generally without medical co-morbidities.

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INTRODUCTION

According to the American Cancer Society, approximately 9610 new cases of testicular cancer will be diagnosed in 2020 with 440 deaths[1]. One in every 250 males will develop testicular cancer during their lifetime[1,2]. With the emergence of cisplatin-based chemotherapy in the 1970's[3], patients with testicular cancer are able to attain a 5-year survival rate of more than 95%[1,4,5]. However, the risk of a thromboembolic event (TEE) poses a significant concern in this population who are usually young- and middle-aged adults without medical co-morbidities. Several factors predispose to experiencing a TEE in the testicular oncologic setting including bulky retroperitoneal disease leading to vascular compression and stasis, endothelial damage resulting from vascular invasion, platinum-based chemotherapy with cisplatin and bleomycin that causes alterations of the clotting system, cigarette smoking, stress, central venous catheter (CVCs), presence of the germ cell tumor marker bhCG that predisposes to thrombosis, and recent surgery that enhances inflammation and disrupts thrombotic and hemostatic pathways[6-11].

The standard chemotherapy regimen is 3 cycles of bleomycine, etoposide, cisplatin (BEP) or 4 cycles etoposide, cisplatin (EP) for good-risk testicular germ cell tumor (TGCT), or 4 cycles of BEP or 4 cycles of vasoactive intestinal peptide (etoposide, ifosphamide, cisplatin) for intermediate- or poor-risk TGCT[5,12,13]. Given the cumulative toxicity of bleomycine, a regimen of 3 cycles of BEP followed by 1 cycle of EP has been utilized in certain patients with intermediate risk. This schedule may be curtailed if a patient experiences a TEE due to safety concerns. Treating a TEE in cancer patients is challenging due to the risk of recurrent TEE with continuation of systemic

chemotherapy and a higher bleeding risk than in patients without cancer[14].

Herein, we present 68 patients with TGCT all of whom underwent cisplatin-based chemotherapy. The factors associated with an increased risk of TEE in this patient population are discussed. An analysis of thromboprophylaxis in the ambulatory setting for patients with high-risk cancer is also presented.

MATERIALS AND METHODS

Study population and data collection

Under an Institutional Review Board-approved protocol, we reviewed the medical records and imaging studies of 68 consecutive individuals who were diagnosed with TGCT and received platinum-based chemotherapy at our Institution between January 1, 2014 and December 31, 2019. All patient in our study underwent orchiectomy (without any exceptions), and the pathology was determined based on the orchiectomy. Standard dosing and cycles of BEP and EP were planned to be completed unless patients experienced complications from chemotherapy. A TEE was defined as a venous or arterial complication that occurred following the patient's orchiectomy for TGCT. A TEE was confirmed by venous Doppler ultrasonography or contrasted computed tomography scans. Patients with a deep venous thrombosis (DVT) had a contrasted chest computed tomography scan at baseline to rule out pulmonary embolism (PE) and to determine the length of anticoagulation treatment. None of the patients was treated with primary thromboprophylaxis.

Statistical analysis

The statistical analysis was performed by stratisfying each metric (TEE, retroperitoneal lymph nodes (RPLN) ≥ 3.0 cm before chemotherapy, age, body mass index (BMI), cigarette smoker, marijuana use, side of orchiectomy, pathologic stage, CVC, type of chemotherapy, number of chemotherapy cycles, RPLN dissection (RPLND), and recurrence after chemotherapy by those patients who experienced a TEE and by those who did not. When comparing the two groups, the Fisher's exact test and the Mann-Whitney *U*-test compared binary/categorical and continuous data, respectively. All analyses were performed using R software (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Demographics and clinicopathologic characteristics

The mean age of the 68 patients with TCGT at orchiectomy was 31 years (16-53 years) (Table 1). The mean BMI at the first medical oncology visit following orchiectomy and before the initiation of chemotherapy was 29 (18.6-58.4). A total of 35 (51%) patients were current or previous cigarette smokers, and 16 (23.5%) patients were current cannabis users.

The majority [49 (72%)] of patients were diagnosed with a non-seminoma and were pathologic stage II (Table 1). A total of 18 (26%) patients had retroperitoneal lymph nodes RPLN ≥ 3.0 cm before chemotherapy, and a slightly higher number of TCGT were on the left [35 (52%)]. Most patients were treated with 3 cycles of BEP. A total of 18 (26.5%) patients underwent a RPLND, consisting of 6 before chemotherapy and 12 afterwards. RPLND is an option prior to chemotherapy in cases of mixed germ cell tumors with negative tumor markers and evidence of retroperitoneal adenopathy. RPLND is a viable possibility especially if a teratoma is suspected, the tumor is not bulky, or if the patient prefers to avoid chemotherapy. Only 1 patient had a family history of testicular cancer, specifically, in a paternal uncle. One patient was treated with 17 fractions of 2250 cGy radiation after the orchiectomy and prior to the chemotherapy.

Five patients experienced recurrent disease necessitating a second course of chemotherapy after their initial cisplatin-based chemotherapy regimen. One of these patients underwent several additional courses of chemotherapy including high dose chemotherapy with autotransplant, however, succumbed to progressive disease 5 years after his orchiectomy. His death was the only one in our study.

Table 1 Characteristics of patients with testicular germ cell tumors at our Institution

Metric		Number of patients (*n* = 68)
Demographics		
Mean age at orchiectomy (yr)		31 (16-53)
Mean BMI at 1st medical oncology visit, *n* (%)		29 (18.6-58.4)
Cigarette smoker or ex-smoker		35 (51)
Current cannabis user, *n* (%)		16 (23.5)
Clinicopathologic characteristics		
Histology, *n* (%)	Seminoma	19 (28)
	Non-seminoma	49 (72)
Pathologic stage, *n* (%)	IA	1 (1.5)
	IB	1 (1.5)
	IS	6 (9)
	IIA	31 (45.5)
	IIB	10 (14.5)
	IIC	8 (11.5)
	III	1 (1.5)
	IIIA	2 (3)
	IIIB	4 (6)
	IIIC	4 (6)
Laterality, *n* (%)	Left	35 (52)
	Right	33 (48)
RPLN ≥ 3.0 cm before chemotherapy, *n* (%)	Yes	18 (26)
	No	50 (74)
Type of chemotherapy	BEP	58 (85.3%)
	EP	4 (5.9%)
	BEP + EP	6 (8.8%)
Cycles of chemotherapy	1	1 (1.5%)
	2	3 (4.4%)
	3	49 (72.0%)
	4	13 (19.1%)
	6	1 (1.5%)
	7	1 (1.5%)
RPLND	Yes	18 (26.5%)
	No	50 (73.5%)
Recurrence after initial chemotherapy	Yes	5 (7.4%)
	No	63 (92.6%)

BMI: Body mass index; BEP: Bleomycin, etoposide, and cisplatin; EP: Etoposide and cisplatin; RPLN: Retroperitoneal lymph node; RPLND: Retroperitoneal lymph node dissection.

Thromboembolic events

A total of 19 (28%) patients experienced a TEE, consisting of 8 PEs, 8 DVTs, and both a PE and DVT in 3 patients (Table 2). Six patients with a TEE had a CVC for delivering chemotherapy. Four of the DVT were peripheral inserted central catheter-line related. Two patients had infusaports, one of whom had no evidence of a fibrin sheath or

Table 2 Thromboembolic events in patients with testicular germ cell tumors who underwent cisplatin-based chemotherapy at our Institution (*n* = 19)

Type/pathologic stage of testicular tumor	RPLN ≥ 3.0 cm before chemotherapy	Type/number of cycles of chemotherapy	PE/DVT	Timing of PE/DVT in relation to chemotherapy	Anticoagulant	Hospitalization
Seminoma IIB	Yes	BEP (2 cycles), EP (1 cycles)	DVT	Same day immediately prior to Cycle 1	Fondaparinux sodium	Yes
Seminoma IIA	No	EP (3 cycles)	DVT[1]	During (Cycle 1)	Heparin → apixaban	Yes
Seminoma IIIB	No	EP (4 cycles)	PE + DVT[2]	3 yr after initial chemotherapy, recurrent disease	Enoxaparin sodium	Yes
Non-seminoma IIB	Yes	BEP (2 cycles)	DVT[2]	During (Cycle 2)	Warfarin →enoxaparin sodium	Yes
Non-seminoma IIB	No	BEP (3 cycles)	PE	During (Cycle 2)	Heparin → enoxaparin sodium → rivaroxaban → apixaban	Yes
Non-seminoma IIC	Yes	EP (4 cycles)	PE + DVT	1 wk before Cycle 1	Heparin (lovenox) → warfarin	Yes
Non-seminoma IIA	No	BEP (3 cycles)	DVT[2]	During (Cycle 2)	Fondaparinux sodium	No
Non-seminoma IIB	Yes	BEP (3 cycles)	PE[1]	During (Cycle 3)	Fondaparinux sodium → warfarin	No
Seminoma IIA	No	BEP (3 cycles)	DVT[2]	During (Cycle 3)	None	No
Seminoma III	No	BEP (3 cycles)	PE	During (Cycle 3)	Enoxaparin sodium	No
Non-seminoma IIIC	Yes	BEP (4 cycles)	PE	During (Cycle 4)	Enoxaparin sodium	No
Non-seminoma IIIC	Yes	BEP (4 cycles)	DVT	During (Cycle 3)	Enoxaparin sodium	No
Non-seminoma IIA	No	BEP (3 cycles)	PE	During (Cycle 3)	Heparin → rivaroxaban	Yes
Non-seminoma IIA	No	BEP (2 cycles)	PE	During (Cycle 2)	Enoxaparin sodium	No
Seminoma IIC	Yes	BEP (3 cycles)	PE	1 mo after chemotherapy ended	Heparin → enoxaparin sodium	Yes
Seminoma IIA	No	BEP (3 cycles)	PE + DVT	During (Cycle 2)	Heparin → enoxaparin sodium	Yes
Non-seminoma IIB	No	BEP (3 cycles)	DVT	1 d prior to Cycle 1	Heparin → fondaparinux sodium	Yes
Non-seminoma IIIA	No	BEP (3 cycles)	PE	2 wk after chemotherapy ended	Warfarin + enoxaparin sodium	No
Seminoma IIC	Yes	EP (4 cycles)	DVT[2]	During (Cycle 2)	Enoxaparin sodium → fondaparinux	Yes

[1]Infusaport.
[2]Peripherally inserted central catheter line. PE: Pulmonary embolism; BEP: Bleomycin, etoposide, and cisplatin; DVT: Deep vein thrombosis; EP: Etoposide and cisplatin.

pericatheter thrombus detected by fluoroscopic evaluation. The other patient had a family history of thromboembolic conditions.

Eight (42%) patients were diagnosed with a seminoma, and patients were either pathologic stage II [14 (74%)] or stage III [5 (26%)] (Table 2). The RPLN were ≥ 3.0 cm before chemotherapy in 8 patients (42%). The relative proportions of TEEs by at least one type stage was significantly different from the other stages (P = 0.05) (Table 3). Patients with stage IS testicular cancer were significantly (P = 0.03) less likely to experience a TEE compared to patients who had stages II or III. Note that no patients with stage IS testicular cancer had a TEE.

Most patients were scheduled to undergo 3 cycles of BEP. The relative proportions of TEEs by at least one type of chemotherapy treatment was significantly different from the other chemotherapy types (P = 0.004) (Table 3). Patients who were treated with BEP/EP and EP were significantly 5-times (1.02-28.3, P = 0.02) more likely to experience a TEE compared to patients who were only treated with BEP.

Table 3 Comparison of metrics between patients with testicular germ cell tumors at our Institution stratified by those who experienced a thromboembolic event and those who did not

	Thromboembolic event		
Metric	**No (%)**	**Yes (%)**	***P* value**
n	49	19	
Age [mean (SD)]	30.9 (7.86)	30.79 (8.67)	0.909
BMI [mean (SD)]	27.87 (6.38)	30.22 (9.25)	0.237
Cigarette smoker = Yes (%)	27 (55.1)	8 (42.1)	0.421
Marijuana use = Yes (%)	13 (26.5)	3 (15.8)	0.526
Side of orchiectomy = Right (%)	27 (55.1)	6 (31.6)	0.107
Pathology = Seminoma (%)	11 (22.4)	8 (42.1)	0.135
Pathologic stage (%)			0.050
I	10 (20.4)	0 (0.0)	
II	33 (67.3)	14 (73.7)	
III	6 (12.2)	5 (26.3)	
Port = Yes (%)	4 (8.2)	3 (15.8)	0.390
Type of chemotherapy (%)			
BEP X 3	45 (91.8)	13 (68.4)	
BEP X 3 + EP X 1	4 (8.2)	2 (10.5)	
EP X 4	0 (0.0)	4 (21.1)	
RPLN ≥ 3.0 cm = Yes (%)	10 (20.4)	8 (42.1)	0.123
RPLND = Yes (%)	12 (24.5)	6 (31.6)	0.555
Recurrence after chemotherapy = Yes (%)	3 (6.1)	2 (10.5)	0.614

BMI: Body mass index; BEP: Bleomycin, etoposide, and cisplatin; EP: Etoposide and cisplatin; RPLN: Retroperitoneal lymph node; RPLND: Retroperitoneal lymph node dissection.

The TEE occurred during the cisplatin-based chemotherapy in 13 (68%) patients. A total of 18 (95%) patients underwent anticoagulant therapy, and 10 (56%) patients were hospitalized for management of their TEE. Eight (42%) patients were current or ex-cigarette smokers, and 3 (16%) were current cannabis users.

One patient sustained bilateral PE and a DVT 3 years after the completion of 4 cycles of EP when he experienced recurrent testicular disease. He was hospitalized and treated with enoxaparin. This patient also experienced recurrent disease and was the sole death in our study.

DISCUSSION

Cisplatin chemotherapy has been reported to cause PE, DVT, myocardial infarction, and peripheral arterial thrombosis[11,15,16]. Several pathogenic mechanisms have been identified between cisplatin and thrombosis, including endothelial cell damage marked by increased plasma levels of Von Willebrand factor during chemotherapy, platelet activation, and up-regulation of prothrombotic factors. Lauritsen *et al*[17] investigated cardiovascular risk factors and disease following BEP treatment for male germ cell cancer[17]. These authors reported that patients had a more than 20-fold increased hazard ratio of venous TEE compared with the normal population which decreased to a hazard ratio of 1.4 after 10 years.

In our literature review the percentage of patients who experienced a TEE with testicular cancer ranged between 5.2% and 19%[6,9,16,18,19]. Most of the TEE occurred during chemotherapy[9,16,18,19]. Various risk factors for a TEE were confirmed, including retroperitoneal and supraclavicular lymph node metastases, elevated serum lactic acid dehydrogenase, higher clinical stage, CVC, high body surface area, and febrile

neutropenia. Paffenholz *et al*[16] reported a significantly reduced overall survival in patients with a TEE during a median follow-up of 8 mo[16]. The present study concurs with this finding, as the TEE occurred during the cisplatin-based chemotherapy in 13 (68%) patients.

The Khorana score is a predictive model for chemotherapy-associated TEE and is based on the following: (1) Site of cancer; (2) Prechemotherapy platelet count 350 × 10^9 /L or more; (3) Hemoglobin level less than 100 g/L or use of red cell growth factors; (4) Prechemotherapy leukocyte count more than 11 × 10^9/L; and (5) BMI 35 kg/m^2 or more[2,20,21]. The risk of TEE is 4-6-fold higher in patients with cancer than in those without, and the TEE incidence is 3%-15% annually in cancer patients[14,18]. The use of thromboprophylaxis to reduce the recurrence of cancer-associated TEE have been examined primarily in cancers such as breast, colorectal, lung, pancreatic, and multiple myeloma that usually affect older individuals[14,21-24].

Carrier *et al*[22] determined that apixaban resulted in a significantly lower rate of venous thromboembolism (VTE) than placebo among intermediate-to-high-risk ambulatory patients with cancer who started chemotherapy[22]. In the intention-to-treat analysis, major bleeding occurred in 10 (3.5%) patients in the apixaban group and in 5 (1.8%) patients in the placebo group (P = 0.046). Khorana *et al*[25] concluded that high-risk ambulatory patients with metastatic cancer who were receiving chemotherapy and were treated with rivaroxaban did not have a significantly lower incidence of VTE or death due to VTE in a 180-d trial period[25]. Despite these two large randomized phase III trials, routine prophylaxis for TEE has not been embraced by clinicians in the outpatient setting as the absolute risk reduction is small and thromboprophylaxis is associated with a heightened risk of major bleeding and high cost[14,18,22].

Of the 574 patients in Carrier and colleagues' placebo-controlled study, only 3 patients were diagnosed with testicular cancer. Khorana *et al*[25]'s study of 1080 patients did not specify the number of patients with testicular cancer, however, mentioned that there were 32 cases of "genitourinary" cancers that included renal, bladder, ureteral, and testicular cancers but not prostate cancer. Young men with TGCT are generally without co-morbidities. This patient population was not represented in large trials investigating thromboprophylaxis in metastatic cancer during chemotherapy. This is largely due to inclusion and exclusion criteria based on the Khorana scoring system used in these trials.

The cisplatin-based chemotherapy used to treat TGCT often does not cause severe thrombocytopenia. A relatively short course of chemotherapy in metastatic testicular cancer often leads to a cure despite the need for ongoing systemic therapy in most other metastatic solid tumors. Additionally, the cisplatin dose in the BEP regimen is higher compared to most regimens used for non-testicular cancer. This important difference may lead to better outcomes with the use of thromboprophylaxis. TGCT possesses numerous risk factors that increase the risk for clotting including bulky retroperitoneal disease, cisplatin-based chemotherapy, cigarette smoking, and possibly marijuana use. Furthermore, the risk of bleeding in TGCT is likely to be lower compared to other types of cancers due to the shorter duration of chemotherapy in TGCT and younger patient population with better bone marrow function. These factors support further investigation into the use of thromboprophylaxis as patients receive chemotherapy for TGCT.

We propose conducting a multicenter randomized double blind placebo control trial examining the efficacy and safety of thromboprophylaxis in patients undergoing systemic treatment for metastatic TGCT. Such a trial will use a novel inclusion scoring system specific for TGCT that incorporates risk factors often encountered with this cancer. Carrier *et al*[22]'s as well as Khorana *et al*[25]'s studies only included patients who had 2 risk factors on the Khorana scale[22,25]. Most of the patients who experienced a TEE in the present study would not have fulfilled the criteria for inclusion for thromboprophylaxis in these 2 trials. The new TGCT scoring system should incorporate the following risk factors: Higher pathologic stage and greater number of chemotherapy cycles as confirmed by the current study as well as elevated serum lactate dehydrogenase, CVC, large RPLN, and seminoma[6,9,16,18,19].

Several studies have reported the limited use of thromboprophylaxis in TGCT with cisplatin-based chemotherapy (Table 4). Paffenholz *et al*[16] reported that 93% of all of their TCGT received prophylactic anticoagulation which is customary in Germany[16]. Thromboprophylaxis during chemotherapy for TGCT is not customary in the United States.

Kentucky has both the highest incidence and death rates for lung cancer in the United States[26,27]. In 2017, a total of 24.6% of adults in Kentucky smoked while the national average was 14.0%[26,28]. Cigarette smoking is a well-recognized risk factor for both atherosclerotic disease and TEE[29]. Half of the patients in our study were either

Table 4 Patients with testicular germ cell tumors who underwent cisplatin-based chemotherapy and experienced a thromboembolic event in the literature

Ref.	Number of patients	Percentage of patients with TE	Main findings
Piketty *et al*[9], 2005	100 patients with GCT; 100 controls with various neoplasms	19 (19%)	> All stages of TGCT; > TE: 1st day of chemo to 6 mo after; > Of 19 TEE, 14 occurred during chemotherapy, 5 after chemo; > Risk factors for TEE: High body surface area, elevated serum LDH
Honecker *et al*[6], 2013	193	22 (11%)	> All stages of TGCT; > TEE: Considered therapy-associated if occurred from start of chemotherapy to 6 wk after; > 18 (%) TEE occurred before chemotherapy; > Risk factors for TEE: Pure seminoma, retroperitoneal or supraclavicular lymph node metastases, elevated LDH, CVC
Bezan *et al*[18], 2017	657	34 (5.2%)	> All stages of TGCT; > TEE: During 1st year of follow-up; > Risk factors for TEE: Higher clinical stage and RPLN, increased number of cycles of chemotherapy
Tran *et al*[19], 2019	1135	150 (10%)	> Metastatic TGCT; > TEE: During or within 90 d of chemotherapy; > Risk factors for TEE: Large RPLN, CVC
Paffenholz *et al*[16], 2019	225	49 (19%)	> All stages of TGCT; > TEE: Start of chemotherapy to 6 mo after; > Risk factors for TEE: Higher clinical stage, elevated serum LDH, febrile neutropenia, CVC; > Patients with TEE had significantly reduced overall survival during median follow-up of 8 mo
Current Study, 2020	68	18 (26.5%)	> All stages of TCGT; > TEE: Following orchiectomy; > Risk factors for TEE: Higher pathologic stage, greater number of chemotherapy cycles

TGCT: Testicular germ cell tumor; TEE: Thromboembolic event; LDH: Lactic acid dehydrogenase; RPLN: Retroperitoneal lymph node; CVC: Central venous catheter.

current or previous cigarette smokers, and 42% of these patients experienced a TEE while undergoing cisplatin-based chemotherapy. Cannabis is the most commonly used recreation drug in the United States and has been suggested to cause myocardial infarction and ischemic stroke[30]. Only a single case of PE in a young male following heavy cannabis use has been reported[30]. It has been suggested that cannabinoids, the active component of cannabis, cause endothelial cell disruption leading to vascular thrombosis. A total of 23.5% of the patients in our study were current cannabis users, and 16% of the cannabis users suffered a TEE while undergoing chemotherapy.

Our study of TCGT offers several unique aspects that shed light on the risk factors for a TEE and educational opportunities for preventing a TEE. Our study, consisting of a single Institution in a metropolitan community in Kentucky, has a large number of patients with TCGT over a 6-year period. The high percentage (28%) of patients who experienced a TEE compared to the literature may be attributed to numerous cumulative factors leading to a hypercoagulable state that may provoke a TEE, including bulky retroperitoneal disease, higher pathologic stage, greater number of chemotherapy cycles, metastatic disease, the remarkably large number of patients who smoke cigarettes and use cannabis, high number of patients with CVC, and lack of patients who were treated with thromboprophylaxis. The limitation of the present study is its retrospective nature and limited number of patients.

Several risk factors have been identified as increasing the risk of developing VTE, including the patient's age (> 60 years), obesity, and history of anterior VTE[31]. The tumor's site, histological type, and stage also elevate the risk of VTE. Pancreatic cancer is the solid tumor with the highest likelihood of VTE, while lymphoma, acute leukemia, and multiple myeloma represent hematologic malignancies that pose a strong risk of VTE. Adenocarcinomas have a higher risk of VTE compared to squamous cell carcinomas, and an advanced tumor stage increases the prospect of developing a VTE[31]. Advanced tumor stage and use of subclavian catheters are the main risk factors for CVC-associated thrombosis[32]. Our Institution generally does not use CVC for delivering chemotherapy in patients with TCGT. However, CVC placement may be unavoidable for patients with poor venous access or patient preference. Educating patients and providers about the thromboembolic risk of CVC may discourage them from selecting CVC as the desired route of chemotherapy infusion. Further educational opportunities with patients that may decrease the likelihood of TEE and enhance overall medical health include an emphasis on discontinuing cigarette smoking and cannabis use as well as promoting weight loss.

CONCLUSION

Although patients with TGCT are a relatively healthy population due to their young age, myriad risk factors such as bulky retroperitoneal disease, higher clinical stage, CVC, cigarette smoking, and cannabis use may heighten their risk of developing a TEE during cisplatin-based chemotherapy. Patients with TGCT who experience a TEE may be unable to complete the course of prescribed chemotherapy despite initiating treatment with an anticoagulant. There may be a significant increase in cost of care following a TEE, including hospital stay, potential requirement for an aggressive procedure, and need for a longer duration of anticoagulation. Patients who could not complete the course of prescribed chemotherapy due to TEE will likely need more frequent medical oncology appointments with monitoring tumor markers as well as closer follow-up imaging studies which can substantially escalate the cost of patient care. High-risk ambulatory patients with TGCT treated with cisplatin-based chemotherapy may benefit from treatment with primary prophylactic anticoagulation to mitigate the potential morbidity and mortality associated with a TEE. Multi-institutional randomized studies are warranted to evaluate the safety and efficacy of thromboprophylaxis during chemotherapy for TGCT in this young patient population.

ARTICLE HIGHLIGHTS

Research background

Cisplatin-based chemotherapy has significantly increased the survival of patients with testicular germ cell tumor (TGCT), although it is associated with a high rate of thromboembolic events (TEE).

Research motivation

As TGCT is the most curable solid tumor and most common cancer among men 18-39 years, we sought to evaluate our single-center experience of patients who were diagnosed with TGCT and received platinum-based chemotherapy. Patients who suffered a TEE were the primary focus of this study.

Research objectives

Our objective was to identify patients who were diagnosed with TGCT and received platinum-based chemotherapy, with particular attention to those patients who experienced a TEE.

Research methods

The medical records and imaging studies of 68 consecutive individuals who were diagnosed with TGCT and received platinum-based chemotherapy at our Institution in a metropolitan community between January 1, 2014 and December 31, 2019 were reviewed. Statistical analysis was performed by stratisfying each metric (TEE, retroperitoneal lymph nodes ≥ 3.0 cm before chemotherapy, age, body mass index, cigarette smoker, marijuana use, side of orchiectomy, pathologic stage, central venous

catheter, type of chemotherapy, number of chemotherapy cycles, retroperitoneal lymph nodes dissection, and recurrence after chemotherapy by those patients who experienced a TEE and by those who did not.

Research results

A total of 19 (28%) patients experienced a TEE following orchiectomy which occurred during chemotherapy in 13 (68%) of these patients. Patients with a higher pathologic stage (stage III) were significantly (P = 0.023) more likely to experience a TEE compared to patients who had a lower stage. Patients who were treated with 3 cycles of bleomycine, etoposide, and cisplatin and 1 cycle of etoposide and cisplatin or 4 cycles of etoposide and cisplatin were significantly 5 (P = 0.02) times more likely to experience a TEE compared to patients who were treated with only 3 cycles of bleomycine, etoposide, and cisplatin.

Research conclusions

Since myriad factors predispose to a TEE such as large retroperitoneal disease, higher clinical stage, greater number of chemotherapy cycle, central venous catheter, cigarette smoking, and possible cannabis use, high-risk ambulatory patients with TGCT treated with cisplatin-based chemotherapy may benefit from prophylactic anticoagulation.

Research perspectives

Randomized studies to evaluate the safety and efficacy of prophylactic anticoagulants are essential in this young patient population generally lacking medical co-morbidities.

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GUIDELINES

GOECP/SEOR radiotherapy guidelines for thymic epithelial tumours

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Abstract

Thymic epithelial tumours (TET) are rare, heterogeneous neoplasms that range from resectable indolent tumours to aggressive thymic carcinomas with a strong tendency to metastasize. The pathological diagnosis is complex, in part due to the

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existence of several different classification systems. The evidence base for the management of TETs is scant and mainly based on non-randomised studies and retrospective series. Consequently, the clinical management of TETs tends to be highly heterogenous, which makes it difficult to improve the evidence level. The role of technological advances in the field of radiotherapy and new systemic therapies in the treatment of TETs has received little attention to date. In the present clinical guidelines, developed by the GOECP/SEOR, we review recent developments in the diagnosis and classification of TETs. We also present a consensus-based therapeutic strategy for each disease stage that takes into consideration the best available evidence. These guidelines focus primarily on the role of radiotherapy, including recent advances, in the management of TETs. The main aim of this document is to promote the standardisation of clinical practice and lay the foundations for future studies to clarify the main unresolved questions related to the optimal management of TET.

Key Words: Targeted therapies; Thymic epithelial tumors; Radiotherapy; Reirradiation; Guidelines; Chemotherapy

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Core Tip: Thymic epithelial tumours (TET) are rare, heterogeneous tumours. The clinical management of these tumours is largely based on studies with a low level of evidence. For this reason, it is especially important to develop guidelines to help guide the optimal approach to managing this disease. The present clinical guidelines review the main recommendations for the diagnosis and classification of TET, providing recommendations for the therapeutic approach based on the tumour stage and pathological anatomy. An important focus of these guidelines is the role of radiotherapy and novel therapies.

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INTRODUCTION

Thymic epithelial tumours (TET) are a group of rare, heterogeneous thoracic tumours originating in the thymus gland. They account for 20% of mediastinal tumours and up to 30% of all anterior mediastinal masses in adults. The thymus gland is composed of two identical lobes involved in the process and maturation of T lymphocytes. At birth, the thymus gland weighs approximately 15 g, reaching its maximum size during puberty (40-45 g). Later, the gland undergoes a physiological involution and atrophies in adulthood, where the gland consists mainly of adipose tissue with scattered lymphocytes, weighing only 5 g in older people. Macroscopic or microscopic thymic tissue remains have been described in other locations between the hyoid bone and the diaphragm. This could be due to variations in the migration of the embryonic endodermal epithelium from the third pharyngeal pouches[1].

The World Health Organization (WHO) classification divides TETs into three histological types: Thymomas, thymic carcinomas, and thymic neuroendocrine tumours[2]. Together, these three types of TETs represent less than 1% of all neoplasms. Thymomas are the most common type of TETs, with an annual incidence ranging from 0.13 to 0.32 cases *per* 100000 people. By contrast, thymic carcinomas, with an annual incidence of 0.02-0.05 *per* 100000 people, account for only 10%-15% of TETs[3-5].

Thymomas are usually slow-growing, indolent tumours. The most common presentation is an incidental finding on imaging tests. They tend to be locally invasive. The most common route of dissemination is the pleura, pericardium, and diaphragm; extrathoracic metastases are very rare[6].

Thymic carcinomas are aggressive tumours with a worse prognosis than other TETs. Their behavior pattern is similar to other tumours that share the same histology. Most thymic carcinomas are diagnosed in advanced stages, presenting lymphatic and/or hematogenous metastases in 10% to 30% of cases[7]. Thymic neuroendocrine tumours should be considered a distinct clinical entity and its management is not covered in the present guidelines.

The incidence rate for TETs is similar in men and women. The peak incidence rate occurs in the 6th decade of life, although the age of presentation may be earlier in patients with myasthenia gravis (MG)[4,8]. Genetic risk factors, such as multiple endocrine neoplasia 1, could influence the development of thymomas and thymic carcinomas[9]. Environmental or infectious factors have not been shown to play a role in the pathogenesis of TETs. However, there does seem to be an association between thymomas and systemic diseases such as MG (the most commonly associated illness), pure red-cell aplasia, hypogammaglobulinemia, and systemic lupus erythematosus. Some hematopoietic cancers (such as leukaemia or diffuse large B-cell lymphoma), as well as other solid tumours (colon, stomach, pancreas, and thyroid) occur more frequently in patients diagnosed with thymoma[10].

METHODOLOGY: LEVELS OF EVIDENCE

These guidelines have been prepared by radiation oncologists with extensive expertise in the management of thoracic tumours. All the physicians in this expert group are members of the GOECP of the SEOR.

These guidelines are based on a review of the most relevant studies published in high-impact, peer-reviewed, indexed journals, together with a review of the clinical guidelines and recommendations produced by the major scientific societies.

The Medline database was searched for relevant systematic reviews, clinical guidelines, and primary studies up to September 2020. Search terms included 'thymoma', 'thymic carcinoma', 'TET', 'thymic neoplasms', 'thymectomy', 'guidelines', 'International Thymic Malignancy Interest Group (ITMIG)', 'staging', 'postoperative radiotherapy (PORT)', 'radiation therapy', 'chemotherapy', 'proton', 'recurrent', 'reirradiation', 'targeted therapies', 'immunotherapy'.

To define the levels of evidence and degrees of recommendation, we used the classification of the Standard Operating Procedures for authors and templates for the European Society of Medical Oncology (ESMO) Clinical Practice Guidelines and ESMO-Magnitude of Clinical Benefit Scale Scores, adapted from the system of the American Society of Infectious Diseases[11,12].

The levels of evidence and grades of recommendation are defined in Table 1.

DIAGNOSIS AND STAGING

Diagnosis

The diagnostic process requires an appropriate differential diagnosis to rule out other types of anterior mediastinal masses, including lymphomas, teratomas, seminomas and non-seminomatous germ cells tumours[13]. A complete, meticulous medical history should be performed, including a comprehensive physical examination (especially neurological). The appropriate complementary examinations (imaging studies and blood tests) should also be performed.

TETs can have a variable form of presentation. Approximately one-third of patients are asymptomatic at diagnosis[10]. The most common clinical manifestations include compressive thoracic symptoms (chest pain, cough, dyspnea, dysphagia, superior vena cava syndrome) and/or symptoms derived from related autoimmune disorders, mainly MG[4]. MG is more common in type AB, B1 and B2 thymomas and much less common in thymic carcinomas[14,15]. Approximately 25%-45% of patients diagnosed with thymoma have MG, although only 15%-20% of patients with MG develop thymoma[14,16,17].

The imaging test of choice is chest computed tomography (CT) with intravenous contrast, which enables a complete evaluation of the mediastinum and pleura from the apex to the costodiaphragmatic recesses (IVA)[18]. CT imaging is comparable or superior to magnetic resonance imaging (MRI) for the diagnosis of masses in the anterior mediastinum, except for cystic lesions (IVB)[13,19].

Table 1 Levels of evidence and grades of recommendation

Levels of evidence	
I	Evidence from at least one large RCT of good methodological quality (low potential for bias) or meta-analyses of well-conducted randomised trials without heterogeneity
II	Small or large RCTs with suspicion of bias (lower methodological quality) or meta-analyses of such trials with demonstrated heterogeneity
III	Prospective cohort studies
IV	Retrospective cohort studies or case-control studies
V	Studies without control group, case reports, expert opinions
Grades of recommendation	
A	Strong evidence for efficacy with a substantial benefit, strongly recommended
B	Strong or moderate evidence for efficacy but with a limited clinical benefit, generally recommended
C	Insufficient evidence for efficacy or benefit does not outweigh the risk or the disadvantages (adverse events, costs, *etc*.), optional
D	Moderate evidence against efficacy or for adverse outcome, generally not recommended
E	Strong evidence against efficacy or for adverse outcome, never recommended

RCT: Randomised controlled trial.

In terms of laboratory tests, a complete blood analysis is advisable, including hemogram and biochemistry, as well as the assessment of biomarkers (beta-human chorionic gonadotropin and alpha-fetoprotein) for the differential diagnosis with germ cell tumours[13].

The use of 18^{F}-fluorodeoxyglucose (FDG) positron-emission tomography-computed tomography (PET/CT) is not recommended for use in routine diagnostic imaging. However, this imaging modality may be useful to complete the imaging study to assess distant disease if dissemination is suspected. 18^{F}-FDG PET-CT may be especially valuable during follow-up if there are doubts about the persistence or recurrence of the disease. A recent meta-analysis highlighted an association between types B3 and C (thymic carcinoma) and increased uptake on 18^{F}-FDG PET-CT, although it should be noted that thymic hyperplasia can also present elevated FDG uptake[20].

Table 2 Lists the most common clinical manifestations of TET, as well as the most characteristic clinical criteria and signs to establish the differential diagnosis with other anterior mediastinal masses[4,10,13,14,16-18,20].

If the diagnostic tests and imaging studies suggest a high probability of a clinical diagnosis of thymoma, and if the lesion is resectable (no break in continuity with the thyroid gland), then no preoperative biopsy is necessary (IVE). In all other cases, a histological sample must be obtained, preferably by CT-guided core needle biopsy or surgical incisional biopsy by mediastinotomy or thoracoscopy (IVD). Whenever possible, transpleural access should be avoided due to the risk of pleural dissemination[21].

Classification

Thymomas are the most common TET subtype. Pathologically, they are classified into types A, A/B, B1, B2, and B3, in addition to several newer, less frequent subtypes. Use of the term "benign thymoma" is discouraged, as aggressive behaviour can never be completely ruled out. In some cases, it can be highly challenging to accurately determine the pathological type. In the 4th edition of the WHO classification system, updated in 2015, the histological criteria were refined, and parameters based on immunohistochemical characteristics introduced. These changes led to a more reproducible and robust classification, thus facilitating diagnosis of thymomas with an uncertain histology (Table 3)[2,5,22].

Thymic carcinomas have several histological subtypes, as follows: Squamous carcinoma (the most common), basaloid carcinoma, mucoepidermoid carcinoma, lymphoepithelioma-like carcinoma, clear cell carcinoma, sarcomatoid carcinoma, adenocarcinoma, "NUT" carcinoma, and undifferentiated carcinoma[2].

Neuroendocrine tumours include small-cell neuroendocrine carcinomas, large-cell neuroendocrine carcinomas, and carcinoids with neuroendocrine differentiation. Neuroendocrine markers include: Chromogranin-A, synaptophysin, CD-56, and neuronal-specific enolase[2].

Table 2 Characteristic clinical manifestation of thymomas and differential diagnosis with other causes of mediastinal masses

	Speed of development	Associated clinical manifestations	Characteristic signs
Thymoma	Long course or indolent	Compressive symptoms: Chest pain, dyspnea, dysphagia, vena cava syndrome	Laboratory abnormalities: Anti-acetylcholine receptor antibodies (common if associated with MG), hypogammaglobulinemia, erythropenia, pancytopenia
		Neurological autoimmune disorders: MG, myotonic dystrophy, limbic encephalitis, peripheral neuropathy	
		Hematologic autoimmune disorders: Red-cell aplasia, pernicious anemia, erythrocytosis, pancytopenia	
Thymic carcinoma	Rapid growth	Collagen autoimmune disorders: Systemic lupus erythematosus, rheumatoid arthritis, Sjogren's syndrome, scleroderma	PET-CT: Thymoma types A, AB, B1-2: Low uptake.
		Endocrine disorders: Multiple endocrine neoplasia, Cushing's syndrome, thyroiditis	PET-CT: Type B3 thymoma and thymic carcinoma: High uptake, with loss of bilobar structure of the thymus
		Autoimmune deficiencies: Hypogammaglobulinemia, T-cell deficiency	
Lymphoma	Fulminant onset	Dermatological disorders: Pemphigus, Lichen planus	Elevated LDH
		"B" symptoms: Fever, weight loss, sweating	
Teratoma	Slow development	Lymphadenopathy. Asymptomatic or compressive symptoms of long duration	CT: Heterogeneous mass, with cystic component and fat/calcifications
Germ cell tumors	Rapid development	Testicular mass	Seminoma: Elevated beta-HCG
			No seminoma: Elevated beta-HCG and AFP
Thymic hyperplasia	Indolent	Asymptomatic	PET-CT: Elevated uptake in mass that maintains the thymic bilobar structure

MG: Myasthenia gravis; PET-CT: Positron emission tomography-computed tomography; LDH: Lactate dehydrogenase; HCG: Human chorionic gonadotropin; AFP: Alpha fetoprotein.

Staging

The classification of TETs by clinical or tumour stage is based on the pathological and surgical criteria first proposed in 1978 and published by Masaoka *et al*[23] in 1981 and modified by Koga in 1994 (Table 4) (IIIA). The Masaoka-Koga classification system is the most widely used as it correlates pathologic and surgical findings with overall survival (OS)[23,24].

The International Association for the Study of Lung Cancer (IASLC), together with the ITMIG, jointly developed a tumor-node-metastasis (TNM) classification system based on an international retrospective analysis of more than 10000 cases (Table 5)[25]. This classification system emphasises the degree of invasion of adjacent structures to determine levels of involvement. This system is highly useful to assess tumour resectability. Levels T1-3 indicate invasion of structures susceptible to surgical resection, while level T4 indicates the involvement of unresectable structures. In addition, this classification system deemphasises features not considered clinically relevant, such as whether the tumour is encapsulated or not[26].

The IASLC-ITMIG system has been incorporated into the 8th edition of the American Joint Committee on Cancer-TNM classification as the official classification system for TETs, offering a universal, standardised system with a consistent nomenclature. As this system becomes more widely used, it should further improve our understanding of this disease[26-30].

TREATMENT OF THYMOMAS

The management of TETs is complex and not without controversy, mainly due to the rarity and heterogeneous nature of these tumours. In addition, the recent introduction of the new staging system (the aforementioned TNM developed by the IASLC/ITMIG) may generate confusion when comparing studies that use different staging criteria[29,30]. The Masaoka-Koga stage classification system remains the most widely

Table 3 Diagnostic criteria of thymomas in the 2015 World Health Organization classification of thymic tumors

Thymoma subtype	Obligatory criteria	Optional criteria
Type A	Occurrence of bland, spindle-shaped epithelial cells (at least focally); paucity[1] or absence of immature (TdT+) T cells throughout the tumor	Polygonal epithelial cells; CD20+ epithelial cells
Atypical type A variant	Criteria of type A thymoma; in addition, comedo-type tumor necrosis; increased mitotic count (> 4/2 mm^2); nuclear crowding	Polygonal epithelial cells; CD20+ epithelial cells
Type AB	Occurrence of bland, spindle-shaped epithelial cells (at least focally); abundance of immature (TdT+) T cells focally or throughout tumor	Polygonal epithelial cells; CD20+ epithelial cells
Type B1	Thymus-like architecture and cytology: Abundance of immature T cells, areas of medullary differentiation (medullary islands); paucity of polygonal or dendritic epithelia cells without clustering (*i.e.*, < 3 contiguous epithelial cells)	Hassall's corpuscles; perivascular spaces
Type B2	Increased numbers of single or clustered polygonal or dendritic epithelial cells intermingled with abundant immature T cells	Medullary islands; Hassall's corpuscles; perivascular spaces
Type B3	Sheets of polygonal slightly to moderately atypical epithelial cells; absent or rare intercellular bridges; paucity or absence of intermingled TdT+ T cells	Hassall's corpuscles; perivascular spaces
MNT	Nodules of bland spindle or oval epithelial cells surrounded by an epithelial cell-free lymphoid stroma lymphoid stroma	Lymphoid follicles; monoclonal B cells and/or plasma cells (rare)
Metaplasticthymoma	Biphasic tumor composed of solid areas of epithelial cells in a background of bland-looking spindle cells; absence of immature T cells	Pleomorphism of epithelial cells; actin, keratin, or EMA-positive spindle cells
Rare others[2]		

The atypical type A thymoma variant is the only new addition to the "historic" list of thymoma subtypes.
[1]Paucity versus abundance: Any area of crowded immature T cells or moderate numbers of immature T cells in more than 10% of the investigated tumor are indicative of "abundance".
[2]Microscopic thymoma, sclerosing thymoma, lipofibroadenoma.
MNT: Micronodular thymoma with lymphoid stroma.

Table 4 Clinical stage of thymic epithelial tumours according to Masaoka-Koga

Stage	Diagnostic criteria	10-yr survival
I	Tumours encapsulated macroscopically and microscopically (without capsular invasion)	84% (81%-86%)
II	A: Microscopic transcapsular invasion	83% (79%-87%)
	B: Macroscopic invasion of fatty tissue or pleural and/or pericardial adhesion	
III	Macroscopic involvement of adjacent structures (pericardium, great vessels, or lung). A: With invasion of great vessels	70% (64%-75%)
	B: Without invasion of great vessels	
IV	A: Pleural or pericardial spread	42% (26%-58%)
	B: Hematogenous or lymphogenic metastasis	53% (32%-73%)

used system and it has been employed for this guidelines[23,24,26].

Numerous clinical guidelines and recommendations from expert working groups are available. However, the recommendations in these documents are based mainly on data from non-randomised trials and small, retrospective case series[18,31-33]. Due to the lack of high-level scientific evidence, many recommendations are based exclusively on expert opinion. In this context, all cases should be referred to a multidisciplinary tumour board for clinical decision-making.

Surgery

In early-stage disease, complete surgical resection is achieved in nearly all cases, with negligible mortality rates. Consequently, surgery is the treatment of choice for stages I, IIa, and IIb (IVA).

The aim of surgical treatment should be complete resection of the tumour, the residual thymus, and the perithymic fat (IVC). In the case of thymomas, unlike thymic carcinomas, thymectomy should not include systematic lymphadenectomy due to the minimal risk of nodal metastasis. Nodal sampling could be considered in more locally-advanced stages or less favourable histologies, although there is a lack of clear

Table 5 Clinical stage of thymic epithelial tumours: International Association for the Study of Lung Cancer/International Thymic Malignancy Interest Group tumor-node-metastasis

Stage		Description
Primary tumor (T)		
T1	T1a	Encapsulated or unencapsulated, with or without extension into mediastinal fat
	T1b	Extension into mediastinal pleura
T2		Direct invasion of the pericardium (partial or full thickness)
T3		Direct invasion of the lung, brachiocephalic vein, superior vena cava, chest wall, phrenic nerveand/or hilar (extrapericardial) pulmonary vessels
T4		Direct invasion of the aorta, main pulmonary artery, myocardium, trachea, or esophagus
Lymph nodes (N)		
N0		No nodal involvement
N1		Anterior (perithymic) lymph nodes (IASLC levels 1, 3a, 6 and/or supradiaphragmatic/inferior phrenic/pericardial)
N2		Deep cervical or intrathoracic nodes (IASLC levels 2, 4, 5, 7, 10 and/or internal mammary nodes)
Distant metastasis (M)		
M0		No pleural, pericardial, or distant metastases
M1	M1a	Pleural or pericardial nodules
	M1b	Pulmonary intraparenchymal nodule or distant organ metastasis
TNM stage		MASOKA-KOGA stage
I	T1N0M0	I, IIA, IIB, III
II	T2N0M0	III
IIIA	T3N0M0	III
IIIB	T4N0M0	III
IVA	Any T, N0-1, M0-1a	IVA, IVB
IVB	Any T, N0-2, M0-1b	IVB

IASLC: International Association for the Study of Lung Cancer; TNM: Tumor-node-metastasis.

evidence to support this decision.

The standard surgical approach is median sternotomy (IVA). Minimally-invasive techniques, such as video-assisted thoracoscopy or robotic surgery, are not usually recommended due to the lack of mature data in TETs[18,31]. However, in the hands of an expert surgeon, these techniques could be considered in stages I and II provided that complete oncological resection can be assured[34-36].

In stage III disease, the use of minimally-invasive surgery is discouraged due to the lack of long-term data and the difficulty of achieving complete resection in cases that require resection of the pericardium, phrenic nerve, pleura, lung parenchyma, and even vascular structures. During thymectomy, the pleural surface must be carefully checked for the presence of metastatic implants, which, if present, should be resected if possible[18,31].

In the case of extensive tumours with a high risk of close surgical margins or residual tumour, the placement of surgical clips to guide PORT is highly recommended.

Radiotherapy

Adjuvant PORT is not indicated in completely resected early-stage tumours (IIE). A key trial randomised patients with stage I disease (n = 29) to surgery or surgery plus adjuvant PORT, with significantly better 10-year survival rates in the group treated with surgery alone (92% *vs* 88%)[37].

An analysis by the Surveillance, Epidemiology, and End Results group evaluated the role of adjuvant radiotherapy in 901 patients with thymomas treated with surgical resection between the years 1973 and 2005. The results of that analysis showed that PORT did not provide any clinical benefit in stage I disease: The cause-specific survival rate at 5 years was 98% with surgery alone *vs* 91% in the group that received adjuvant PORT ($P = 0.03$)[38].

In stage II TETs, there is some controversy between clinical guidelines and the recommendations of expert working groups. The ESMO guidelines recommend considering radiotherapy in patients with unfavourable histologies (*e.g.*, B3), but that recommendation is not included in other guidelines such as the Italian collaborative group for thymic malignancies (TYME) or the recommendations of a Canadian group by Falkson *et al*[33], who consider that there is insufficient evidence to recommend PORT in stage II disease due to the toxicity risk. Falkson and colleagues recommend discussing the advantages and disadvantages of this option with the patient. However, some groups still recommend considering PORT after complete resection in cases with unfavourable histologies (IVC)[18,32,33].

Several studies have shown that adjuvant radiotherapy provides some survival benefits in stage IIB and III disease, increasing 5-year survival rates from 66% to 76% ($P = 0.01$)[38]. The value of adjuvant radiotherapy in patients with higher tumour stages may be that higher stages have lower complete resection (R0) rates, with R0 rates of 47% in stage III disease and 26% in stage IV disease[39-41].

Adjuvant radiotherapy is indicated in most patients who undergo resection for stage IIB, III, and IV thymoma. However, this indication is not absolute as the evidence to support this approach is limited and the reported results are sometimes contradictory[38,42-47].

Therapeutic recommendations

Below we provide treatment recommendations for thymomas according to disease stage using the Masaoka-Koga classification shown in Figures 1 and 2.

Stage I: Biopsy not indicated (IVE).

The treatment of choice is surgical resection (IVA).

Complete resection (R0): No adjuvant treatment (IIE)[42,43].

Incomplete resection (R1): PORT (50-54 Gy) (IVB).

Stage IIA: Biopsy not indicated (IVE).

The treatment of choice is surgical resection (IVA).

Complete resection (R0): A-B2: No adjuvant treatment (IVC); B3: Consider PORT (45-50 Gy) (IVC).

Incomplete resection (R1): PORT (50-54 Gy) (IVB).

Stage IIB: Biopsy not indicated (IVE).

The treatment of choice is surgical resection (IVA).

Complete resection (R0): A-B1: No adjuvant treatment (IVC); B2-B3/pericardium+: Consider PORT (45-50 Gy) (IVC).

Incomplete resection (R1): PORT (50-54 Gy) (IVB).

Stage III-IVA: Resectable tumour. The treatment of choice is surgical resection (IVA). After surgery, administer PORT (IVB). The recommended dose is 45-50 Gy for R0, with a boost to 54 Gy for R1 or 60-70 Gy for R2[38,48-51].

Unresectable tumour. A biopsy should be performed to confirm the diagnosis of thymoma and to rule out thymic carcinoma. Initiate anthracycline/cisplatin-based induction chemotherapy (ChT). After ChT, re-evaluate tumour resectability with imaging tests[48-51].

If the tumour is resectable after ChT: Surgical resection (IIIA) + PORT (IVB). The recommended dose is 45-50 Gy for R0; 54 Gy for R1; 60-70 Gy for R2. In cases of R2, assess concomitant platinum/etoposide-based chemoradiotherapy (CRT) (IVB).

If the tumour is unresectable after ChT: Radical radiotherapy (60-70 Gy) with or without concomitant platinum/etoposide-based ChT (IVB)[18].

Stage IVB: The treatment of choice is chemotherapy (IIIA).

If the patient is in good general condition [performance status (PS), 0-1], the use of anthracycline-based ChT regimens is recommended.

The most common regimen is capecitabine (cyclophosphamide + doxorubicin + cisplatin) for a maximum of 6 cycles (IIIB).

Imaging re-evaluation (using the same imaging tests used for diagnosis) is recommended mid-treatment (VA)[52].

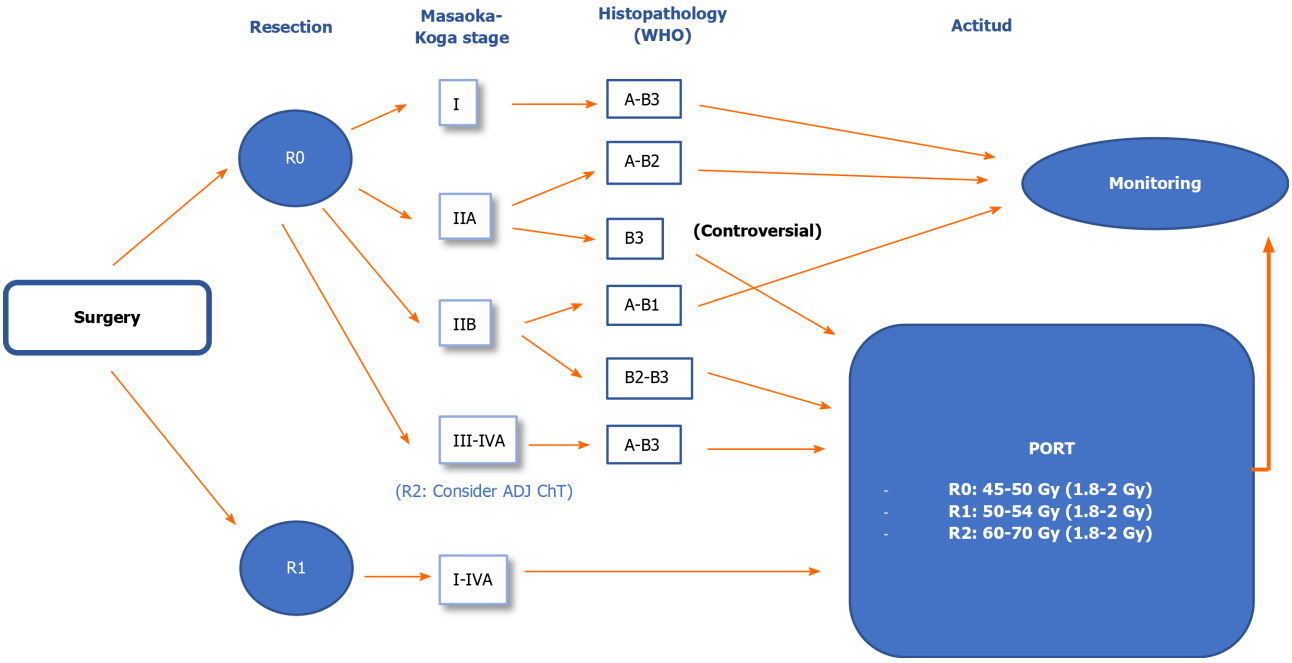


Figure 1 Therapeutic algorithm for resectable thymomas. PORT: Postoperative radiotherapy; ADJ ChT: Adjuvant Chemotherapy.

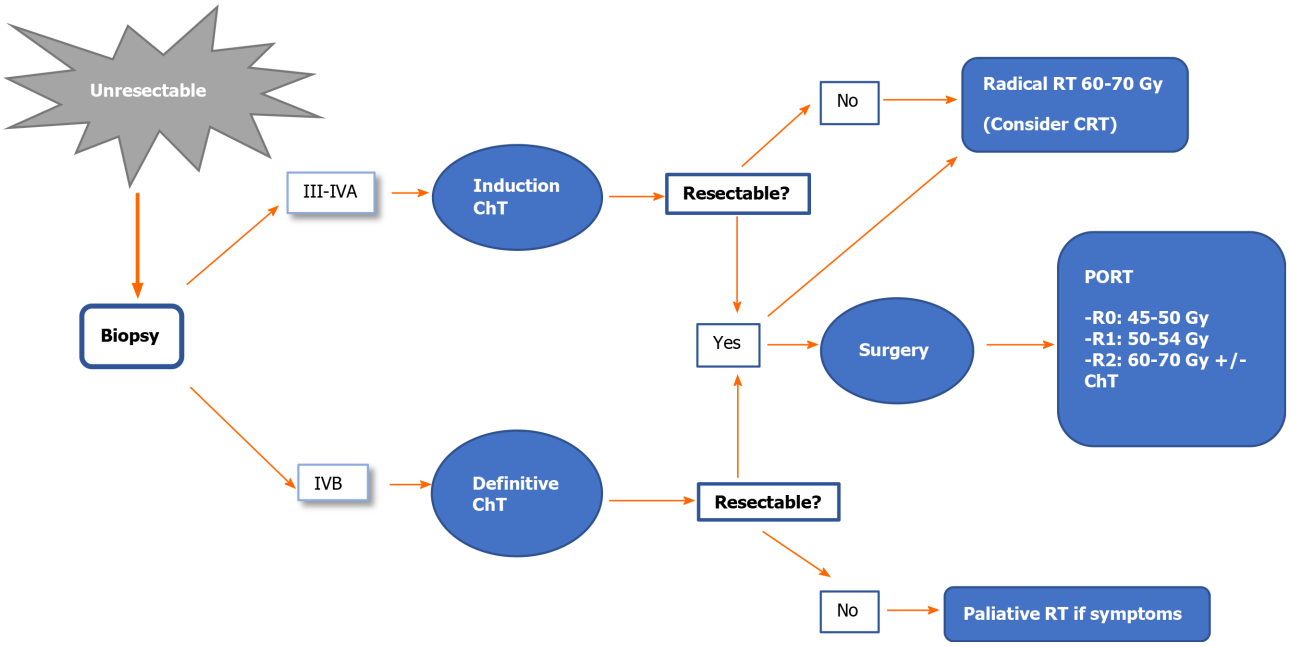


Figure 2 Therapeutic algorithm for unresectable thymomas. ChT: Chemotherapy; RT: Radiotherapy; CRT: Chemoradiotherapy. PORT: Postoperative radiotherapy.

In allergic patients or patients with poor PS, carboplatin-paclitaxel ChT could be considered[53].

If the tumour is resectable after ChT, assess surgery and PORT, or radical radiotherapy (IVC).

Debulking surgery is not recommended[32].

In advanced stages, palliative radiotherapy for pain relief or decompressive radiotherapy could be considered in cases with severe vascular involvement.

TREATMENT OF THYMIC CARCINOMA

Thymic carcinoma is a very rare but aggressive tumour commonly diagnosed in advanced stages. These tumours have the capacity to metastasize to the lymph nodes and even to extrathoracic regions[54,55]. Thymic carcinoma has a worse prognosis than thymoma, mainly related to tumour resectability and stage. The 5-year survival rates are 90% in stage I-II disease and 28% in stages III-IV[56].

Thymic carcinoma differs from thymoma in terms of the histologic, immunohistochemical and genetic characteristics. The new WHO classification clearly identifies thymic carcinoma as a distinct entity. Determination of the immunohistochemical parameters is often required for the differential diagnosis[5]. It is important to differentiate thymic carcinomas from primary squamous cell lung cancers that have metastasized to the thymus, since these tumours sometimes have histological characteristics that are similar to those of thymic carcinoma[57].

As with thymomas, clinical staging of thymic carcinoma is essential to determine the appropriate treatment[33]. However, given the rarity of this tumour and the lack of data from prospective, randomised studies to define the optimal strategy, there is no consensus regarding the ideal therapeutic approach. Given the aggressiveness of these tumours, multimodal treatment involving multidisciplinary teams is common.

Complete surgical resection is an independent prognostic factor and a key treatment recommendation (IVA)[58]. ITMIG/IASLC guidelines define complete resection as complete resection of the tumour, residual thymus, the surrounding fat, and lymphadenectomy[59,60].

Therapeutic recommendations

Below we provide treatment recommendations for thymic carcinoma according to disease stage using the Masaoka-Koga classification (Figures 3 and 4).

Stage I: Surgical resection is the treatment of choice (IVA).

Complete resection (R0): No additional treatment required.

Incomplete resection (R1): PORT (50-54 Gy) (IVB).

Stage II: Surgical resection is the treatment of choice (IVA).

Complete resection (R0): Consider PORT (45-50 Gy), especially for stage IIB (IVB).

Incomplete resection (R1): PORT (50-54 Gy) (IVB). Postoperative ChT should be considered.

Stage III-IVA: Resectable tumour. Surgical resection (IVA) followed by PORT (IB).

The recommended doses are 45-50 Gy, 54 Gy, and 60-70 Gy for R0, R1 and R2 resections, respectively.

In R0 resections, postoperative ChT is optional; in R1-R2 cases, postoperative ChT is recommended[61].

Unresectable tumour. The diagnosis of thymic carcinoma should be confirmed by biopsy. 2-4 cycles of induction ChT followed by imaging studies to reassess tumour resectability are recommended[48-51]. Various ChT schemes are available based on cisplatin (IIIA) combined with other agents such as etoposide (IIIB), doxorubicin, or cyclophosphamide[18,34].

If the tumour is resectable after ChT: Surgery (IIIA) followed by PORT (in R0, 45-50 Gy; in R1, 54 Gy) (IVB). Consider postoperative ChT.

If the tumour is unresectable after ChT or the resection is classified as R2, the treatment indication is radiotherapy (60-70 Gy) with or without concomitant ChT (IVB)[18].

Stage IVB: The treatment of choice is cisplatin-based ChT (IIIA). Various ChT regimens are available[18]. Table 6 shows the most common schemes.

Due to advances in the molecular characterisation of TETs in recent years, our understanding of the tumour response to targeted agents has improved substantially, especially in thymic carcinomas. Several signalling pathways have been evaluated as potential targets, including KIT, vascular endothelial growth factor receptor (VEGFR), and mammalian target of rapamycin (mTOR).

In chemotherapy-refractory thymic carcinoma, the targeted agent imatinib may be a treatment option, although this drug is not recommended in patients without a KIT mutation (IVB)[62]. Sunitinib may also be an alternative in refractory tumours, regardless of KIT status (IIIA)[63]. Everolimus can also be considered in treatment-refractory cases (IIIB)[64].

Table 6 Most commonly used chemotherapy regimens for thymic carcinoma

Scheme	Drugs
ADOC	Doxorubicin + Cisplatin + Vincristine + Cyclophosphamide
CAP	Cisplatin + Doxorubicin + Cyclophosphamide
PE	Cisplatin + Etoposide
VIP	Etoposide + Ifosfamide + Cisplatin
CODE	Cisplatin + Vincristine + Doxorubicin + Etoposide
Carbo-Px	Carboplatin + Paclitaxel
CAP-GEM	Capecitabine + Gemcitabine

ADOC: Anthropogenic dissolved organic carbon; CAP: Capecitabine; PE: Pulmonary embolism; VIP: Vasoactive intestinal peptide; GEM: Gemcitabine.

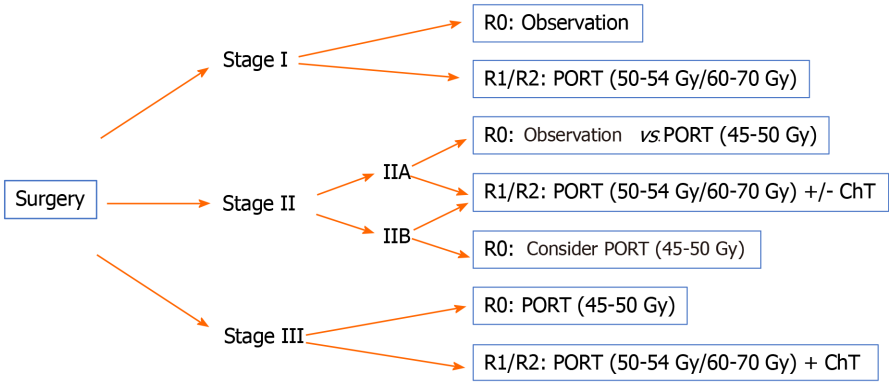


Figure 3 Therapeutic algorithm for resectable thymic carcinomas. PORT: Postoperative radiotherapy; ChT: Chemotherapy.

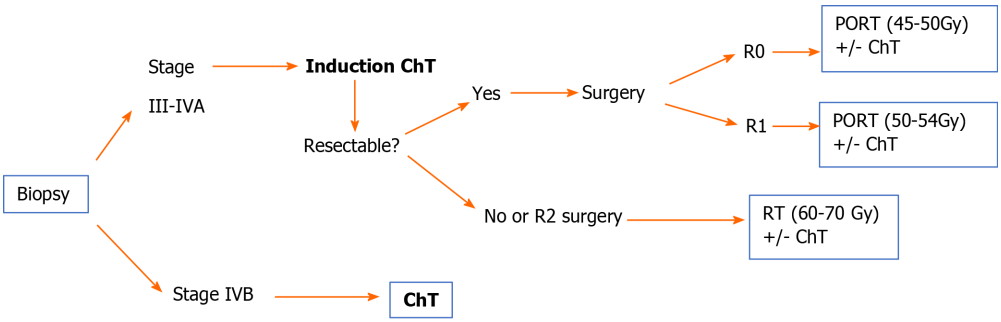


Figure 4 Therapeutic algorithm for unresectable thymic carcinomas. PORT: Postoperative radiotherapy; ChT: Chemotherapy; RT: Radiotherapy.

RADIOTHERAPEUTIC TREATMENT OF TET

Standardisation of the treatment approach is important to enable the comparison of results among different centres, which is why it is important to reach a consensus with regard to target volumes, techniques, and treatment schemes. In turn, this will allow us to develop clinical-therapeutic guidelines for TETs.

In terms of the radiotherapeutic treatment of TETs, various different approaches are used, ranging from preoperative radiotherapy alone, or radiotherapy combined with ChT, and PORT (the most common approach), in which the surgical margin status (R0, R1 or R2) is a crucial factor to select the radiation dose or the need for radical treatment, in which radiotherapy will be administered alone or concomitantly with ChT as the only treatment for the thymic tumour.

Patient positioning

The patients should preferably be immobilized in the supine position with the neck

extended and the arms above the head. A contrast-enhanced CT should be performed to help identify neighbouring structures and to assess postsurgical changes (CT slice thickness: 3-5 mm).

If four-dimensional (4D)-CT is available, acquisition of the full respiratory cycle will improve volume delimitation by determining tumour motion with respiration (internal margin), thus allowing for smaller margins than conventional CT[65]. PET/CT fusion (18^{F}-FDG PET/CT) may be useful in certain circumstances to improve volume definition[66].

Volume definition

Gross tumour volume (GTV): The GTV, if present, is the identifiable macroscopic disease.

Clinical target volume (CTV): Defined as the GTV plus microscopic disease. The incorporation of preoperative imaging tests, as well as pathological findings for the surgical margins, will help define these limits.

Internal target volume (ITV): This is obtained by adding the internal motion of the lesion (internal margin) to the CTV.

Planning target volume (PTV): Defined as the ITV plus systematic uncertainties for each treatment unit (set-up margin).

Depending on the planning technique, daily verification, patient imaging control (IGRT), tumour location, and differences in routine clinical practice, the margins may vary, as follows[67]:

GTV to CTV: 0.5 to 1 cm.

CTV to PTV (conventional CT): 1 to 1.5 cm.

ITV to PTV (4D-CT without IGRT): 0.5 to 1 cm.

ITV to PTV (4D-CT and IGRT): 0.5 cm.

Organs at risk and dose limits

Given the thoracic localization of TETs, it is essential to pay careful attention to the radiation doses to the surrounding organs at risks (OARs), particularly the lungs, esophagus, heart, spinal cord, trachea, and great vessels. The proposed dose limits for the main OARs are shown in Table 7[68].

Irradiation techniques

When evaluating the role of radiotherapy in TETs, it is important to consider that most of the available evidence is based on large retrospective series and multicentre databases. A common limitation of these studies is high variability in diagnostic and treatment standards — including radiotherapy — which makes comparative analyses difficult. In addition, rapid technological progress in the field of radiotherapy has significantly improved treatment precision while simultaneously reducing the associated toxicity. However, the clinical impact of these advances in the treatment of TETs has received scant attention to date[69]. Nevertheless, given the mediastinal location of these tumours, experience and lessons learned from treating other thoracic cancers, such as lung cancer or malignant pleural mesothelioma, can likely be extrapolated to TETs.

Intensity-modulated radiation therapy (IMRT) is the standard radiotherapy modality today due to improved dose conformity to the target volume and lower doses to healthy tissues. If IMRT is unavailable, 3D conformal radiotherapy (3D-CRT) is an acceptable alternative[70]. A retrospective study compared IMRT/3D-CRT to conventional RT found that better survival outcomes in patients treated with IMRT/3D-CRT due to the smaller radiation field, increased precision, and lower doses to healthy organs[71].

The combination of IMRT and 4D-CT simulation could reduce radiation-induced toxicity, with better local control and OS[72,73]. Given the location of these tumours in the thorax, coordinated breathing control techniques could be beneficial in selected patients[65].

Due to the proximity of critical organs (spinal cord, heart, lungs, and esophagus), the risk of toxicity secondary to treatment — especially cardiovascular toxicity — must be carefully considered. One study assessed a consecutive cohort of 74 patients with thymoma treated with surgery plus adjuvant radiotherapy, finding that cardiovascular disease (CVD) was the primary cause of non-cancer mortality, accounting for 50% of deaths. The median time from treatment to CVD diagnosis was 101 mo. In that study, the mean heart dose was an independent risk factor for CVD[74].

It is important to underscore the increased risk of second malignant tumours (particularly esophageal and lung cancer, non-Hodgkin's lymphoma, and soft tissue

Table 7 Constraints to the main organs

Organ	Constraints		
Spinal cord	Dmax < 45 Gy		
	Dmax < 40 Gy if 3 Gy/fraction		
Lung (total lung minus GTV; solely total lung for postoperative cases without GTV)	Mean dose < 20 Gy		
	Mean dose < 8 Gy if post-pneumonectomy		
	RT alone	RT with chemotherapy	Neoadjuvant treatment before surgery
	V20 ≤ 40%	V20 ≤ 35%	V20 ≤ 30%
		V10 ≤ 45%	V10 ≤ 40%
		V5 ≤ 65%	V5 ≤ 55%
	V20 < 10% and V5 < 60% if post-pneumonectomy		
Heart	Mean dose < 26 Gy		
	V30 ≤ 45%		
Esophagus	Mean dose < 34 Gy		
	Dmax ≤ 80 Gy		
	V70 < 20%		
	V50 < 50%		
Kidney	20 Gy < 32% bilateral kidney		
Liver	Mean dose < 30 Gy		
	V30 ≤ 40%		

GTV: Gross tumour volume; RT: Radiotherapy.

sarcomas) in patients with thymoma. These second tumours may be related to the combination of immune dysregulation, and genetic or environmental factors[75-77]. This association has important implications, as irradiation of healthy tissues can promote the development of second tumours due to genetic damage; in addition, if chest reirradiation is required, there is an increased risk of toxicity due to overlapping fields.

IMRT improves dose conformity and distribution in the target volume; however, due to the physical properties of photons, it may not be possible to adequately reduce the dose to the OARs. This has led some authors to propose proton therapy as an alternative to limit the radiation dose to healthy tissues; predictive models suggest that this approach would reduce the risk of acute and late toxicity and second neoplasms[78-81].

A study published in 2018 performed a dosimetric comparison for advanced radiotherapy techniques in 10 surgically-treated patients with thymoma. Dosimetric plans were prepared for 3D-CRT, volumetric modulated arc therapy (VMAT), tomotherapy, proton therapy, and carbon ion therapy. The results showed that heavy particle radiotherapy significantly reduced radiation doses to the OARs compared to photon radiotherapy. In terms of target coverage, the best results were achieved with tomotherapy, although this also resulted in higher low doses to the OARs. The dosimetric parameters for VMAT were superior to those obtained with 3D-CRT[82]. Another study, published in 2019, compared the dosimetric characteristics of VMAT to proton therapy in 20 cases. While the two techniques yielded similar results in terms of coverage, doses to healthy organs were lower with proton therapy[81].

Despite the potential advantages of proton radiotherapy, the clinical evidence to support this approach in TETs is scant. However, one prospective series of 27 patients treated with proton therapy reported a local control rate of 100% and a 3-year OS rate of 94% at a median follow-up of 2 years. Toxicity was mild to moderate with no grade 3 or higher events[83]. Therefore, although proton beam radiation therapy has a clear dosimetric benefit, and theoretical models support a secondary clinical benefit, long-term prospective studies are still needed to compare this radiotherapy modality to other advanced radiotherapy techniques, such as tomotherapy and VMAT.

RECURRENT DISEASE

Disease recurrence is not uncommon in TETs, ranging from 7.5% to 36% of cases. However, in cases with complete surgical resection (R0), the recurrence rate is less than 15%[45,84]. In stage I and II disease, the relapse rate is only 5%-10%; by contrast, in more advanced stages, the recurrence rate is approximately 30% and some cases series have reported relapse rates > 50%[45,85].

Histology is a prognostic factor for recurrence, which can be as high as 28.6% in type B3. Thymic carcinoma is more aggressive than thymoma, with relapse rates greater than 50%[61]. Tumour size is another possible prognostic factor[86] . The mean time to recurrence is 5.5 years (range, 2 to 16)[84].

Locoregional recurrence is the most common pattern of recurrence after complete surgical resection, although thymic carcinomas tend to progress distantly[84,85,87]. There is some evidence suggesting that some thymomas can become more aggressive subtypes when they recur[87].

Treatment of recurrent disease

The management of recurrent disease in TETs should be individualized based on the resectability of the recurrent lesion(s), prior treatments received, and time interval since treatment. These patients should be included in clinical trials whenever possible.

Recurrences should be managed according to the same strategy used to treat the primary tumour (IVA). Thus, surgical salvage with complete resection is the treatment of choice for both local and regional recurrences (isolated pleural metastases)[33,88]. The available data indicate that surgery is superior to non-surgical treatment in resectable recurrences, with 10-year survival rates of around 65% after a second resection[85,87]**.** In patients with multiple pleural metastases, neoadjuvant ChT followed by surgical resection is worth considering when feasible[33].

In patients not previously treated with radiotherapy, the indication is the same as in newly-diagnosed tumours: PORT in patients with subtotal resections or stage III/IVA disease with complete resection (consider in stage II). Radiotherapy can be administered as definitive treatment for unresectable tumours or inoperable cases, potentially with concomitant ChT (IIIA)[18].

Historically, thoracic reirradiation has been limited to palliative doses due to the potential for toxicity related to the proximity of OARs. Cardiac toxicity merits special attention given the tumour localization, patient age, and the frequent use of cardiotoxic chemotherapy agents. However, reirradiation is increasingly being considered due to continuous technological advances in radiotherapy, which allows for increasingly conformal radiation dose delivery.

When considering reirradiation, it is essential to evaluate the patient's clinical status, prior radiotherapy treatments (total dose, fractionation, treatment volumes, technique used, time elapsed since the initial radiation therapy) and other therapeutic alternatives. The risks and benefits of reirradiation are often equally balanced due to the potential toxicity of the dose and treatment volumes.

The available scientific evidence on reirradiation in thymic cancer is scant, although some studies have reported acceptable tolerance[89,90]**.** Most of the studies involving chest reirradiation have been performed in patients with lung cancer treated with 3D-CRT, IMRT, proton therapy, or stereotactic body radiation therapy. In general, radical doses achieve better disease control than palliative doses, and the best results are obtained in patients with good PS, small reirradiation volumes, recurrence outside the original radiotherapy field, and a longer interval between the two radiotherapeutic treatments[91-95]. By extrapolating these experiences to thymic tumours (in the absence of specific studies in TETs), it appears that reirradiation is safe and feasible in well-selected patients, as long as the appropriate technology is available, and the teams are experienced (IVB).

SYSTEMIC TREATMENT OF ADVANCED DISEASE

Chemotherapy

ChT is typically used to treat recurrent disease that is not suitable for surgical salvage, or to treat advanced metastatic disease. Generally, anthracycline/cisplatin-based chemotherapy is the regimen of choice. However, other first-line regimens include vincristine or cyclophosphamide. If the first-line treatment is effective, it can be re-administered in patients who develop disease progression (IVB), although patients should be closely monitored for cardiac toxicity due to the accumulated dose of

anthracyclines[96]. In thymic carcinomas, carboplatin-paclitaxel ChT is the most widely-accepted first-line chemotherapy approach[31].

The use of successive lines of treatment in patients with progressive disease can prolong progression-free survival. Multiple ChT regimens are accepted, although the most common are carboplatin-paclitaxel, platinum-etoposide, capecitabine-gemcitabine, pemetrexed-etoposide, and 5-FU-leucovorin.

Patients not considered candidates for ChT but who have a positive octreoscan may benefit from treatment with octreotide, with or without prednisone (IIB)[18].

Targeted therapies

In recent years, targeted therapies have been incorporated into the therapeutic arsenal (IIIB)[97]. The identification of molecular alterations in various pathways—KIT signalling, VEGFR, and mTOR—has opened opportunities for the off-label use of targeted therapies in treatment-refractory thymic tumours.

The c-KIT receptor is overexpressed in 80% of thymic carcinomas, although it is mutated in only 9%. The efficacy of tyrosine inhibitors (TKIs) such as imatinib, sunitinib, and sorafenib has been evaluated in numerous studies. Some phase II trials involving imatinib in patients with non-mutated B3 thymoma and thymic carcinoma have found no response (IIIE)[62]. However, other studies have shown that treatment with TKIs in patients with mutated c-KIT can achieve disease control[98].

Sunitinib and sorafenib both act on the VEGFR receptor, with activity in tumour angiogenesis, and both have demonstrated efficacy in the treatment of TETs, regardless of the c-KIT receptor status[99]. Sunitinib has been evaluated in patients with TETs in two phase II trials. In one trial, which included patients with thymic carcinoma or thymoma, response rates were 26% and 6%, respectively[63]. The second trial (KOSMIC) included only patients with thymic carcinoma, achieving response rates of 22%, although disease stabilization was achieved in 70% (IIIA)[100].

Lenvatinib is a second-line TKI with activity on the VEGFR, fibroblast growth factor and platelet-derived growth factors receptors. In the phase II REMORA trial, lenvatinib significantly improved the response rate (up to 38%). At present, lenvatinib is only administered within clinical trials[101]. Other antiangiogenic agents (anti-VEGFR) such as bevacizumab or aflibercept have demonstrated efficacy in thymic tumours[18].

Zucali *et al*[64] conducted a phase II trial in 51 patients to evaluate everolimus, an mTOR inhibitor, reporting a disease control rate of 88%, although this was mainly disease stabilization rather than objective response. However, everolimus has been associated with the emergence of potentially severe pneumonitis. Consequently, more studies are needed to better define the indication for everolimus (IIIB)[64].

Several targeted therapies have shown promising results in patients with thymic tumours, but more studies are needed to better determine the most appropriate treatment type and sequence according to individual pathological and molecular characteristics[18]. Targeted therapies are currently used in clinical trials or as off-label drugs, mainly sunitinib or other TKI/anti-VEGFR agents and everolimus (IIB).

Immunotherapy

Several studies have evaluated the efficacy of anti-PD-1 antibodies, such as pembrolizumab. Giaccone *et al*[102] conducted a phase II trial involving 41 patients with thymic carcinoma, reporting a response rate of 23%, mainly disease stabilization (53%). However, these treatments induce a strong immune system response and were associated with non-negligible toxicity and potentially serious adverse effects (*e.g.*, myocarditis, polymyositis, hepatitis, among others) in up to 15% of patients[102].

A study conducted in Korea in patients diagnosed with thymic carcinoma or thymoma reported results that were similar to those described by Giaccone and colleagues. Disease stabilization was achieved in 72% and 54% of patients with thymoma and thymic carcinoma, respectively; however, a high percentage of patients experienced autoimmune side effects[103].

The efficacy of nivolumab was assessed in phase II trial in Japan in 15 patients with thymic carcinoma. Of these, 11 achieved disease stabilization. However, none of the patients showed an objective response, which led to the premature termination of study recruitment[104].

FOLLOW-UP

At present, there are no prospective data on which to establish recommendations

regarding the optimal post-treatment follow-up protocols for thymic tumours. European guidelines—based on the expert consensus statements and published data on the cumulative incidence of recurrences and the pattern of recurrence—recommend chest CT as the imaging technique of choice for follow-up purposes. The first CT image should be obtained 3-4 mo after surgery (VC)[18].

In patients with complete resection of a stage I/II thymoma, an annual CT scan is recommended during the first five years of follow-up and then every two years thereafter. In stage III/IV thymomas, thymic carcinoma, or after an incomplete (R1-2) resection, CT scans should be performed every 4-6 mo during the first two years and annually thereafter. Follow-up CT scans should be performed for 10-15 years after treatment.

The most recent version of the American guidelines recommend follow-up with contrast-enhanced CT every 6-12 mo for the first 2 years followed by annual CT scans for 5 (thymic carcinoma) or 10 years (thymoma). However, these guidelines do not indicate the recommended duration of follow-up. In some cases, the guidelines suggest that MRI may be a useful alternative to CT scans[33].

The emergence of second tumours and autoimmune diseases has been described during follow-up. Patients with clinical MG (or only anti-acetylcholine receptor antibody positivity) have a higher risk of myasthenic crisis in stressful situations or when certain drugs are administered (VA)[18].

CONCLUSION

TET are rare, heterogeneous tumours. The evidence base to support the diagnosis and therapeutic management of this disease is limited. Some recommendations are based mainly on data from retrospective or non-randomised studies, or even expert opinions (due to the lack of evidence). As a result, disease management is often highly heterogeneous. In recent years, several scientific societies and working groups have sought to standardise the management of TETs. The recent development of a universal, standardised classification system with a consistent nomenclature (IASLC-ITMIG) is expected to reduce variability in the management of these tumours. In addition, new guidelines have been published to provide a clear, schematic approach to treatment. Although these guidelines differ on certain points due to the lack of definitive evidence (*e.g.*, the role of radiotherapy in stage II disease), the overall trend is towards the convergence of recommendations, which should facilitate the performance of multicentre studies and, in turn, raise the level of evidence for the management of TETs.

The present document was developed by the GOECP/SEOR to help unify the management of TET in Spain and to lay the foundations for the creation of a stable working group. We believe that it is crucial to prioritise closer collaboration between centres and the relevant scientific societies in order to carry out prospective studies and randomised clinical trials, particularly to resolve the most controversial aspects of this disease management.

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REVIEW

Oncogenic driver mutations in non-small cell lung cancer: Past, present and future

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Abstract

Lung cancer, of which non-small lung cancer is the most common subtype, represents the leading cause of cancer related-death worldwide. It is now recognized that a significant proportion of these patients present alterations in certain genes that drive oncogenesis. In recent years, more of these so-called oncogenic drivers have been identified, and a better understanding of their biology has allowed the development new targeted agents. This review aims to provide an update about the current landscape of driver mutation in non-small-cell lung cancer. Alterations in *Kirsten rat sarcoma, epidermal growth factor receptor, MET, anaplastic lymphoma kinase, c-ROS oncogene 1, v-raf murine sarcoma viral oncogene homolog B, neurotrophic receptor tyrosine kinase, human epidermal growth factor 2, neuregulin-1* and *rearranged during transfection* are discussed, as well as agents targeting these alterations. Current standards of treatment as well as promising future strategies are presented. Currently, more than fifteen targeted agents are food and Drug administration-approved for seven oncogenic drivers in non-small-cell lung cancer, highlighting the importance of actively searching for these mutations. Continuous and future efforts made in defining the biology of each of these alterations will help to elucidate their respective resistance mechanisms, and to define the best treatment strategy and therapeutic sequence.

Key Words: Non-small cell lung cancer; Driver mutations; Tyrosine kinase inhibitors; Targeted agents; Oncogenes

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Core Tip: We have reviewed the current literature about the impact of detecting oncogenic mutations in non-small cell lung cancer (NSCLC). Over the years, the adoption of next generation sequencing has rendered it easier to determine and detect possible oncogenic driver mutations, leading to the development of several targeted therapies. The clinical impact and benefit for patients is important in terms of quality and quantity of life. These therapies are more effective than standard chemotherapy treatment. We have reviewed the data to explain what has been done, is ongoing and shall be done in the future for patients with oncogene-driven NSCLC.

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INTRODUCTION

Lung cancer is the most common malignancy and the leading cause of cancer related deaths worldwide (18.4% of total cancer deaths), with non-small cell lung cancer (NSCLC) being the most common subtype, accounting for approximately 85% of all diagnosed cases[1]. The majority of NSCLC patients display advanced disease when diagnosed and thus have poor prognosis[2]. It is well established that acquired genetic alterations in certain driver genes result in tumor growth and invasiveness, and that patients harbouring certain mutations may benefit from targeted therapies[3] (Figure 1). Indeed, a randomized clinical trial reported that advanced NSCLC patients harbouring activating mutations in the *epidermal growth factor receptor* (*EGFR*), one of the major oncogenic drivers of NSCLC, exhibited longer progression-free survival (PFS) when treated with a tyrosine kinase inhibitor (TKI), gefitinib, compared to those treated with standard platinum-based chemotherapy[4]. However, those who were treated with TKI drugs can acquire secondary resistance mutations, in which case a new treatment regimen is needed to maintain therapeutic effects[5,6]. In addition to *EGFR*, NSCLC patients carrying *anaplastic lymphoma kinase* (*ALK*) or *c-ROS oncogene 1* (*ROS1*) rearrangement were shown to respond well to a different TKI drug[7-9], crizotinib, while *v-raf murine sarcoma viral oncogene homolog B* (*BRAF*) mutated NSCLC patients can be treated with a combination of BRAF inhibitors, dabrafenib and trametinib[10]. These findings suggest that the identification of mutation profiles of NSCLC is critical in order to prescribe suitable TKI therapy as well as elucidate the molecular basis of drug resistance to provide timely treatment adjustment. Since 2018, the American Society of Clinical Oncology (ASCO) has recommended routine mutation testing for driver genes including *EGFR*, *ALK*, *ROS1* and *BRAF* in clinical practice for patients with metastatic NSCLC. Although there are currently no targeted drugs for *Kirsten rat sarcoma* (*KRAS*) or *neuroblastoma rat sarcoma* (*NRAS*) mutated NSCLCs[11,12], mutation testing for these genes has also been recommended due to their proven impact on clinical outcomes of NSCLC patients[13]. This review aims to provide an update about the impact and importance of detecting mutations in oncogenes in patients with advanced NSCLC.

KRAS

The *rat sarcoma* (*RAS*) genes (*KRAS*, *NRAS*, *Harvey rat sarcoma viral oncogene homolog*) represent the most frequent human oncogenes. Up to 30% of NSCLC harbor a mutation in the *KRAS* oncogene, making KRAS the most commonly detected oncogenic driver in lung cancer[11]. The KRAS proteins belong to the small guanosine triphosphate (GTP)ase family, involved in intracellular signaling. In response to extracellular signaling, KRAS proteins switch between two states: The GTP-bound "on-state" and general dental practitioner-bound "off-states". When "on", KRAS activates downstream signaling pathways, mainly the mitogen activated protein kinase and extracellular signal regulated kinase (MAPK/ERK) and phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) C1 signaling pathways, ultimately promoting cellular division and proliferation.

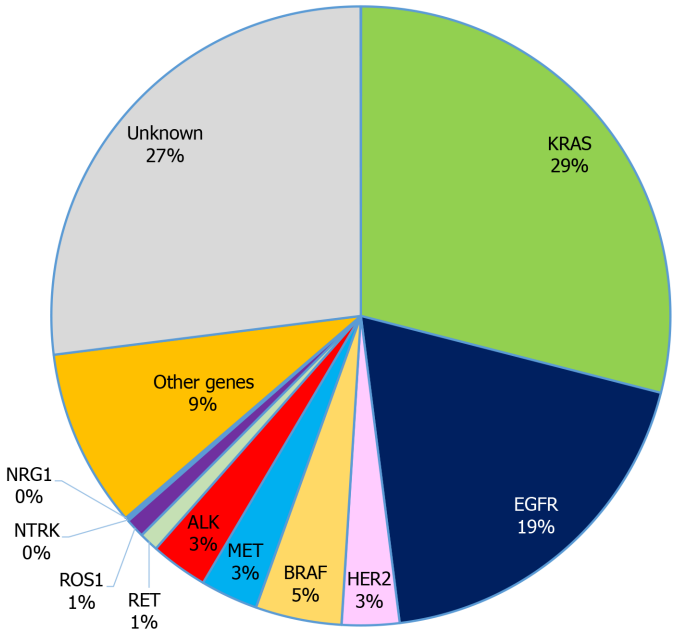


Figure 1 Incidence of oncogenic drivers in non-small cell lung cancer. KRAS: Kirsten rat sarcoma; EGFR: Epidermal growth factor receptor; ALK: Anaplastic lymphoma kinase; HER2: Human epidermal growth factor 2; ROS1: c-ROS oncogene 1; NTRK: Neurotrophic receptor tyrosine kinase; RET: Rearranged during transfection; NRG1: Neuregulin-1.

Some KRAS mutations, such as those in exons 2 and 3, which prevent GTP hydrolysis and prevent switching KRAS signaling off, result in a constitutive activation of KRAS proteins. KRAS mutation is more frequent in adenocarcinoma, and can be detected by next generation sequencing (NGS)[14]. The most common mutations involve a substitution in the codon 12 or 13. The KRAS G12C mutation found in approximately 13% of NSCLC[15], is of particular interest, as it has become a therapeutic target. It is more frequently detected in smokers, while KRAS G12D is more common among non-smokers[11]

Currently, the standard of care for KRAS-mutated NSCLC follows that of non-oncogene-driven NSCLC, consisting of immunotherapy with or without platinum-based chemotherapy.

Two Specific KRAS G12C TKIs have emerged, sotorasib (AMG510) and adagrasib (MRTX849). In the Codebreak100 phase I/II trial, 59 KRAS G12C mutated, previously treated advanced or metastatic NSCLC patients received sotorasib orally. The objective response rate (ORR) was 32.2%, with a median duration of response of 10.9 mo[16].

Sotorasib is currently being tested in the randomized phase III, Codebreak200 trial, *vs* docetaxel in the second-line setting (NCT04303780). The primary endpoint is PFS, with overall survival (OS) as a secondary endpoint[17].

Adagrasib represents another specific KRAS G12C TKI. In the phase I/II KRYSTAL-1 trial[18], 79 patients with pre-treated NSCLC received adagrasib 600 mg twice daily. Among the 51 patients evaluable for response, an ORR of 45% was observed. The most frequent side effects included nausea, vomiting and diarrhea, mostly grade 1-2.

Others KRAS G12C inhibitors are currently being tested in phase I/II clinical trials: JNJ-74699157[19] and Gadolinia-Doped Ceria-6036[20]. As fewer than 50% of patients initially respond to sotorasib or adagrasib, we can assume that some patients present intrinsic resistance to KRAS G12C inhibition. This hypothesis is supported by preclinical evidence demonstrating resistant cell lines[21]. One explanation is that tumor cells may not exclusively rely on the RAS pathway for survival and proliferation[22]. As an example, RAS-independent activation of the PI3K/AKT/mTORC1 signaling pathway could be associated with resistance to KRAS inhibition[23]. Another mechanism of resistance could be the heterogeneous distribution of KRAS mutations in different tumor sites within the same patient[24]. Adaptive resistance also emerges under the selective pressure of KRAS TKIs. One mechanism of adaptive resistance could consist in the amplification of upstream drivers, such as receptor tyrosine kinases/ Src homology 2 domain-containing phosphatase 2 (RTKs/SHP2), that result from KRAS inhibition. Indeed, the diminution of ERK activity driven by KRAS G12C TKIs has been shown to suppress the ERK-mediated feedback inhibition of RTKs/SHP2, further activating N-Ras, H-Ras, and K-RasG12C, and ultimately

restoring the activity of the MAPK/ERK signaling[21,25].

Although clinical data are scarce, it is likely that KRAS G12C inhibitors are not effective in the majority of the patients harboring KRAS G12C mutations. There has been a growing interest to combine the KRAS G12C inhibitors with targeted agents or immune checkpoint inhibitors (ICIs)[26]. Based on preclinical data discussed above, adagrasib is currently being tested in association with the SHP2 inhibitor TNO155 in early clinical phases[27]. Associations with ICIs also represent an interesting approach, as in some preclinical models, KRAS G12C positive tumor cell lines exhibit an immunosuppressive environment that is disrupted by KRAS inhibition[28,29].

Besides those targeting G12C, other KRAS inhibitors are in development, such as MRTX1133, a KRAS G12D inhibitor currently in investigational new drug enabling studies[30], or BI 1701963, a molecule targeting son of sevenless-1, an activator of KRAS, which could allow inhibition of the KRAS pathway regardless of the mutation[31].

Finally, other approaches targeting KRAS include an mRNA vaccine targeting KRAS mutant cells, a strategy that has already entered a phase I clinical trial[32], as preclinical data revealed an immune cell response in animal models[33].

EGFR

EGFR is one of the four members of the human epidermal growth factor (HER) family transmembrane receptors (HER1/EGFR, HER2, HER3, and HER4). Each HER receptor is an inactive monomer that dimerizes with a receptor of the same type or with another member of the HER family in response to ligand binding. The receptor activation triggers a complex downstream signaling network which leads cell replication[34]. The dysregulated receptor function or disruptions in any downstream EGFR processes may result in cell transformation and malignancy.

The prevalence of the mutation in the EGFR oncogenes is 50% among Asian patients with lung adenocarcinoma and 15% among Western patients[35]. Mutations leading to excessive EGFR activity are most common among non-smokers, young, female, Asian lung cancer patients[36]. Exon 19 deletions or L858R point mutations in exon 21 account for 90% percent of the activating mutations in the tyrosine kinase domain of EGFR, resulting in constitutional activation of EGFR without ligand-induced stimulation, thus promoting cell proliferation, survival, and dissemination[37,38].

EGFR mutations can be detected by immunohistochemistry (IHC) or NGS. TKIs are the standard front-line therapy for metastatic EGFR mutant NSCLC for a decade, with three generations of TKIs that demonstrated better outcomes and lower toxicity compared to standard chemotherapy[39], with a median PFS of 11.0 mo (gefitinib or erlotinib) *vs* 5.6 mo (chemotherapy)[40]. To date, there are five United States Food and Drug Administration (FDA)-approved TKIs as the standard treatment for patients with activating EGFR mutations in NSCLC, including first-generation gefitinib and erlotinib, second-generation afatinib and dacomitinib, and third generation osimertinib[41]. However, it has been shown that resistance systematically develops to those treatments[42,43], mediated by mechanisms such as T790M secondary mutations, activation of other EGFR pathways, development of concurrent mutations or histological transformation. The *T790M* exon 20 mutation is rarely found in EGFR TKI-naive disease but is the most frequent cause of resistance to first- and second-generation EGFR TKIs (50%-60% of cases)[44] possibly by the presence of a mutated clone before treatment, which would be free to grow into the dominant clone under selective TKI pressure. It is the indication for which osimertinib, an EGFR-TKI that selectively inhibits both EGFR-TKI-sensitizing and EGFR T790M resistance mutations, was first developed. It improves outcomes in second-line after disease progression with *T790M* mutations[44,45], as well as in the front-line setting (PFS 18.9 mo *vs* 10.2 mo; OS 38.6 mo *vs* 31.8 mo) with an improved safety profile[43,46].

In order to improve outcomes of EGFR mutant NSCLC patients, combination therapy of EGFR TKIs with other agents have been tested, with interesting perspectives. A meta-analysis was conducted regarding the efficacy and safety of vascular EGFR inhibitors in combination with chemotherapy for patients with advanced NSCLC showing improved PFS, ORR and disease control rate, but without an impact on OS[47]. Results are pending from the Japanese phase II study comparing osimertinib alone *vs* osimertinib plus chemotherapy in second-line setting for patients with T790M mutation. Also, the phase III FLAURA2 study started recruiting in 2019, studying osimertinib with or without platinum-pemetrexed chemotherapy as first-line treatment in *EGFR* mutated advanced patientswith NSCLC (ClinicalTrials.gov Identifier: NCT04035486). Numerous studies are ongoing regarding the best sequence

to use. At this time, the combination of chemotherapy and TKIs is not standard practice.

MET

The proto-oncogene *MET* is located on chromosome 7q21-q31. It encodes for a transmembrane receptor (c-Met or MET) also known as hepatocyte growth factor receptor. This tyrosine kinase receptor activates downstream RAS/ERK/MAPK, PI3K/AKT, Wnt/β-catenin, and signal transducer and activator of transcription (STAT) signaling pathways, that can drive cell proliferation, survival, migration, invasion, angiogenesis, and transition from epithelial to mesenchymal[48]. MET dysregulation encompasses a heterogeneous array of alterations, with two main subgroups: MET amplifications and MET exon 14 mutations, leading to prolonged activation of the cellular MET receptor and downstream proliferation pathways[11]. Regarding exon 14, aberrant splicing and skipping of exon in the messenger RNA transcript can result from somatic missense mutations, insertions, deletions, and concomitant insertions and deletions.

MET alterations are found in fewer than 5% of patients with NSCLC, mainly adenocarcinoma, often with concurrent mutations (*i.e.*, EGFR, ALK). Detection of MET amplification is assessed by fluorescence *in situ* hybridization (FISH), and exon 14 skipping is most often completed through DNA or RNA NGS.

Due to the various diversity of MET dysregulation, there was a need to identify the oncogenic role of each type of MET alteration and defining the adequate targeted therapy. Over the last 20 years, several agents have been developed to target MET such as multikinase MET inhibitors (crizotinib, cabozantinib, MGCD265, AMG208, altiratinib, golvatinib), selective MET inhibitors (capmatinib, tepotinib, tivantinib) and monoclonal antibody (onartuzumab, emibetuzumab, ficlatuzumab, rilotumumab). A retrospective registry (IMMUNOTARGET) of advanced NSCLC patients who received immunotherapy showed that only 16% of them demonstrated a partial response, with a short median PFS of 3.4 mo[49]. Small cohorts showed that MET TKIs offer a promising treatment option in patients with exon 14 skipping with response rates from 25% to 68%, and a median duration of response of 9 to 16 mo. In the retrospective data analysis of a phase 1 PROFILE 1001 study, data supported that MET exon 14 skipping in NSCLC confers sensitivity to direct MET inhibitors with a median OS of 24.6 mo *vs* 8.1 mo among patients receiving crizotinib compared with those who did not[50]. In patients with advanced or metastatic NSCLC with a confirmed MET exon 14 skipping mutation, the use of tepotinib, a highly selective oral MET inhibitor, was associated with a partial response in approximately half the patients (ORR 46%, with a median duration of response of 11.1 moin a recent phase 2 study[51]. In advanced MET exon 14 skipping NSCLC, capmatinib showed substantial antitumor activity particularly in previously untreated patients (ORR 68%), with a median duration of response of 12.6 mo[51]. Limited efficacy was observed in previously treated patients with *MET* amplification (ORR 7 to 12% of patients with capmatinib).

ALK

ALK is a transmembrane receptor tyrosine kinase that can activate multiple signaling cascades such as the PI3K-AKT, Crkl-C3G, MAP kinase kinase kinase 2/3-mitogen-activated protein kinase kinase (MEK)5-ERK5, Janus kinase (JAK)-STAT, and MAPK pathways. Its involvement is known in development, then subsequently silenced in adult tissues. However, several *ALK* gene alterations have been identified in tumors, including point mutations, deletions, and rearrangements leading to ALK reactivation. Various ALK-fusion proteins have been described that result from numerous chromosomal rearrangements, with formation of dimers by the amino-terminal portion of the ALK fusion proteins resulting in the activation of the ALK protein kinase domain that plays a key role in the tumorigenic process. The consequent ALK expression can activate multiple downstream known cancer signaling pathways [PI3K/AKT, JAK/STAT, and RAS/rapidly accelerated fibrosarcoma (RAF)/MEK/ERK][52].

ALK rearrangements are detected in approximately 5% of advanced NSCLC[8], 2 to 7% in all, up to 19% for stage IV. ALK alterations are mainly found in adenocarcinomas (97%), while squamous cell carcinomas comprise 3%[7]. ALK positivity is found in a fifth of never to light smokers with lung cancer. Methods of diagnosis can

include FISH, IHC, or NGS.

The superiority of the TKI, crizotinib, over chemotherapy in first-line ALK+ advanced NSCLC was proven in 2014, with an ORR of 74% *vs* 45%, PFS 10.9 mo *vs* 7 mo, and 1-year survival 84% *vs* 79% (median OS not reached in the crizotinib group)[53]. However, resistance to treatment invariably develops, often with progression in the brain, motivating the development of new generations of TKI. Second- (alectinib, brigatinib, ceritinib) and third-generation (lorlatinib) TKIs have proven their efficacy, with the third-generation lorlatinib who led to a 72% improvement in PFS compared with crizotinib in first-line treatment[54]. After failure of second-generation ALK TKIs, concurrent administration of platinum/pemetrexed-based chemotherapy with an ALK TKI shows efficacy in a recent retrospective study[55] (PFS 6.8 mo with the combination *vs* 3.2 mo for chemotherapy alone; suggesting a potential role for continued ALK inhibition. Some studies are currently underway, evaluating the addition of ceritinib to nivolumab (NCT02393625, completion June 2021) or ceritinib with trametinib (NCT03087448, completion June 2022).

Recently, a next-generation ALK inhibitor, ensartinib, demonstrated promising efficacy in the first-line treatment for advanced disease in a preplanned interim analysis (phase III eXalt3 study), with a median PFS of 25.8 mo with ensartinib in the intent-to-treat population *vs* 12.7 mo with crizotinib) (NCT02767804, study completion March 2021). Another new ALK inhibitor, TQ-B3139, is being tested *vs* crizotinib in the first line setting (NCT04009317, completion April 2022).

ROS1

ROS1 encodes a tyrosine kinase receptor, belonging to the insulin receptor family, and structurally related to the ALK protein. Its natural ligand remains undefined.

ROS1 rearrangement was described in glioblastoma, before being recognized as a potential oncogenic driver in NSCLC[56]. Various ROS1 alterations have been described in cancers: overexpression, splice variants (usually leading to a truncated protein lacking the intracellular domain), amplification, mutations and finally fusions with another partner gene. In contrast to fusions, the pathogenic significance of other alterations is undetermined[57].

At the moment, ROS1 fusions have identified with 55 different partners genes in different cancer types, including more than 20 fusion partners in NSCLC. The CD74-ROS1, EZR-ROS1, SDC4-ROS1 and SLC34A2-ROS1 fusion represent the most common rearrangements[58]. All resultant fusion proteins retain an intact ROS1 intracellular kinase domain which, as a result of the rearrangement, becomes constitutively activated. The activated kinase triggers intracellular signaling pathways, such as the RAS–RAF-MEK-ERK, PI3K-AKT-mTOR and JAK-STAT3 pathways, ultimately leading to cellular survival and division.

As with other common driver mutations, patients harboring a ROS1 rearrangement tend to be younger and more frequently non-smokers and Asian[56]. ROS1 is almost always found in adenocarcinoma subtypes, where it represents 1%-2% of the cases[59]. ROS1 alterations rarely occur with other driver mutations[60].

ROS1 rearrangement can be detected by FISH or NGS[14]. IHC is sensitive and can be used as a screening test. However, specificity remains poor and a positive result must be confirmed by FISH assays or NGS. A treatment decision should not be based on IHC results alone.

ROS1 rearranged NSCLC seem to have better response to chemotherapy, especially pemetrexed-based, when compared to both tumors with other driver mutations and wild-type tumors[61]. On the other hand, some data, although scarce, show modest response to ICIs[62].

Several tyrosine kinase inhibitors have shown clinical activity in ROS1-positive NSCLC. The phase I PROFILE 1001 trial of crizotinib included 50 patients with ROS1-fusion NSCLC, among which an overall response rate of 72%, and a median PFS of 19.2 mo were reported[63]. Importantly, more than 80% of patients had received at least one previous line of treatment. An updated analysis reported an impressive median OS of 51 mo[64]. Phases 2 trials in Europe and East Asia have consistently reported similar ORRs of 63%-69%[65,66]. In these studies, crizotinib demonstrated a favorable safety profile. However, crizotinib has limited intracranial activity. The central nervous system represents a common site of progression in ROS1-positive NSCLC treated with crizotinib[67]. Crizotinib should be avoided as a first-line agent in case of untreated brain metastasis.

After progression on crizotinib, lorlatinib represents an option. It was evaluated in 69 patients with ROS1-positive NSCLC[68]. In 40 patients that had received prior crizotinib, the ORR was 35%, with a median PFS of 8.5 mo. In 21 crizotinib-naive patients in the same study, lorlatinib demonstrated an ORR of 62%, with a median duration of response comparable to crizotinib of 25.3 mo and a median PFS of 19.3 mo. Lorlatinib also showed intracranial activity, with intracranial response rates of 50% and 64% in crizotinib pretreated and crizotinib-naïve patients respectively.

Ceritinib is a selective ALK inhibitor that also exhibits activity against ROS1 kinase. In a phase phase II trial involving 32 Asian patients with ROS1-rearranged NSCLC the ORR was 67%[69]. Interestingly, two patients that had received prior crizotinib experienced no response.

Entrectinib represents another tyrosine kinase inhibitor with activity against the ROS1 kinase. In a phase II study among 53 patients with ROS1-positive NSCLC, the majority of which had received previous platinum-based chemotherapy, the ORR reached 77%[70]. Responses seem durable, with median duration of response of 24.6 mo. Intracranial activity was also observed, with an intracranial ORR of 55%, with a median duration of response for central nervous system lesions of 12.9 mo.

Repotrectinib represents a next generation TKI targeting ROS1, ALK and tropomyosin receptor kinase (TRK). Its potency for ROS1 exceeds that of crizotinib by more than 90-fold. Reprotrectinib also shown early signs of efficacy against ROS1 resistance mutations[70,71].

Resistance to TKIs ultimately arise in nearly all patients. Intrinsic resistance to ROS1 inhibition implies solvent-front or gate-keeper point mutations in the ROS1 kinase domain, the most common being the ROS1 G2032R, that precludes binding to crizotinib[72]. To date, more than 20 mutations conferring resistance to various TKI have described[57]. Moreover, resistance to ROS1 inhibition may probably arise from the activation of parallel signaling pathways such as KRAS, BRAF or MET. Emergence of BRAF and KRAS activating mutations and MET-amplification in ROS1 positive NSCLC treated with crizotinib or lorlatinib have been described[73,74]. Upon progression, the role of sequencing ROS1 TKIs remains unclear.

BRAF

BRAF is an intracellular protein kinase that plays a relevant role in the MAPK/ERK pathway, including numerous proteins with kinase domains (RAF, MEK, ERK) that carry signal transduction from membrane receptors to DNA in the nucleus of the cell[75]. BRAF is an oncogene located on chromosome 7 involved in several cell functions, including growth, proliferation, survival and differentiation.

BRAF mutations are found in about about 5.5% of cancers, with almost 200 BRAF mutants and many RAF translocations identified in human cancers[76]. Those alterations generate structural modifications of the protein that are responsible for permanent activation of MAPK pathway and resistance to inhibitory feedback signals.

The frequency of BRAF mutations is about 5 to 8% in lung adenocarcinomas[10], with higher incidence in melanoma (50%), thyroid carcinoma (30% to 70%) and colorectal cancer (5% to 20%). Its presence predicts poor outcome for the latter three. While the V600E activating mutation is the most common BRAF variant found in solid tumors (90%), it only accounts for half of BRAF mutations in NSCLC. Non-V600E variants are more common in males, and all BRAF variant are more common among smokers. It is important to note that clinical characteristics of patients with NSCLC harboring BRAF mutation are difficult to clearly identify due to small numbers of patients in trials.

BRAF mutations can be detected by using immunohistochemistry for V600E exclusively or DNA sequencing on the tumor tissue for the two types.

The efficacy of ICIs is uncertain, and is based on conflicting retrospective data and case series, thus not routinely recommended. Therapies targeting BRAF mutations were developed for melanoma with great success, then used for NSCLC. For lung cancer, numerous small studies tested RAF inhibitors (vemurafenib, dabrafenib) alone or in combination with MEK inhibitors (trametinib, cobimetinib). Best responses were observed when treatment was combined, especially in V600E patients in first-line of dabrafenib-trametinib with a mOS of 24.6 mo[77]. Front-line use of double BRAF/MEK inhibition if V600E mutation is found in advanced NSCLC is now suggested in current guidelines. Other agents such as multikinase inhibitors sorafenib and dasatinib showed responses in case reports[78,79].

Combining MEK and BRAF inhibitors has proven to be more effective than single-agents for the treatment of BRAF-mutant advanced tumors, but does not prevent the

emergence of resistance[79]. Indeed, studies on melanoma describe intrinsic adaptive (other pathways alteration) or acquired (*de novo*) resistance in BRAF mutated cancers treated with targeted inhibitors. Thus, further research is warranted to establish clear therapeutic algorithms.

NEUROTROPHIC RECEPTOR TYROSINE KINASE

The three *neurotrophic receptor tyrosine kinase* (*NTRK*) genes (*NTRK1*, *NTRK2* and *NTRK3*) encode tyrosine kinase receptors for neutrophins, involved in neuronal development, survival and proliferation[80,81].

Oncogenic fusions involving the NTRK genes (*e.g.*, apposition of the 3′ region of the NTRK gene with the 5′ sequence of a fusion partner gene) occur across many cancer types[82]. More than 25 gene partners have been described[83]. Yet, all these fusions result in a constitutively activated and overexpressed TRK kinase, that activates downstream pathways involved in cellular proliferation, such as the MAPK and PI3K/AKT[84].

NTRK fusions are very frequent in a few rare cancer types: Secretory carcinoma, mammary analogue secretory carcinoma, infantile fibrosarcoma and cellular mesoblastic nephromas, in which they are detected in more than of 90% of patients[83]. On the other hand, the NTRK fusion occurs at a very low frequency in various common cancers types, such as colorectal, breast, thyroid and lung cancers. In the latter, it represents under 1% of cases[85,86].

NTRK fusion can be detected either by IHC, FISH or DNA-and RNA-based NGS. Pan-TRK IHC has been shown to have high specificity and sensitivity in the detection of fusion protein expression. RNA-based NGS is preferred to DNA-only based technique.

The European Society for Medical Oncology (ESMO) guidelines suggest using FISH testing (or real-time reverse transcription polymerase chain reaction) in tumors with highly prevalent NTRK fusions, as it probably represents the most cost-effective strategy. In situations where such alterations are uncommon, NGS technique should be used upfront if available or as a confirmatory test, after a positive IHC "screening test". Using NGS upfront has the advantage to allow the detection of other potentially targetable molecular alterations.

Larotrectinib is an oral TRK inhibitor that has shown durable activity in NTRK positive advanced tumors. In an analysis of three phase I/II trials, the ORR among patient with TRK-fusion positive cancer was 79%, with 80% of responses ongoing at 12 mo[15]. Among 12 patients with lung cancer, a similar response rate was reported (75%). The most frequent grade 3-4 adverse events related to larotrectinib were increased alanine aminotransferase, anemia, and decreased neutrophil count. Grade 3-4 adverse events occurred in 46% of patients.

Entrectinib represents another TRK inhibitor, also active for ROS1 and ALK, and specifically designed to cross the blood-brain barrier[87]. Entrectinib demonstrated an ORR of 57% in small non-randomized studies, among 54 patient with TRK-positive advanced tumors, including 10 patients with NSCLC (ORR 70% in the latter)[87]. The median overall duration of response was 10 mo. Of note, an objective intracranial response rate of 54.5% was reported in a cohort of 12 patients with NTRK fusion-positive tumors with brain metastasis[88]. The most common adverse events reported with entrectinib (> 20% of patients) were fatigue, gastro-intestinal disorders, weight gain and cognitive impairment. While the most threatening serious adverse events consisted of congestive heart failure, QT prolongation, central nervous system effects, hepatotoxicity, and vision disorders.

Based on these results, both larotrectinib and entrectinib have been granted accelerated approval by the FDA for metastatic or unresectable NTRK-fusion positive solid tumors, that have progressed following treatment or have no satisfactory standard therapy[89,90].

Despite a prolonged response, resistance to larotrectinib and entrectinib is expected to emerge in most patients[83], *via* different mechanisms: Solvent-front mutations and xDFG substitution[91]. Larotrectinib (LOXO)-195 and repotrectinib represent second-generation TRK-inhibitors, currently under development capable to overcome resistance to first-generation of TRK-inhibitors. LOXO-195 is a highly selective inhibitor of all 3 TRK kinases. LOXO-195 or selitrectinib was evaluated in a phase I trial and a FDA expanded access single patient protocol, including 31 patients in total, all of whom had received prior treatment with a TRK inhibitor[92]. Of note, 7 patients were pediatric. The most frequent treatment-emergent adverse events were dizziness (65%), ataxia (60%), nausea (50%), vomiting (40%), anemia (30%) and gait disturbance

(30%), with 5 double-lumen endobronchial tube (4 ataxia/dizziness) in total. Among the 29 patients evaluable for efficacy, an ORR of 34% was reported.

Repotrectinib[91] is another second-generation NTRK inhibitor, designed to overcome acquired resistance mutations to larotrectinib/entrectinib[93,94] Case reports showed partial response in patient presenting acquired resistance to larotrectinib or entrectinib[87]. Repotrectinib is currently being tested in phase I/II clinical trial (NCT03093116)[95].

HER2

The *HER2*/neu gene is located on the chromosome 17q12, and encodes the HER2 protein, which belongs to the epidermal growth factor receptor (ERBB) family of tyrosine kinase receptors. Other members of the ERBB family include EGFR (ERBB-1), HER3 (ERBB3), and HER-4 (ERBB4). The ERBB receptors are transmembrane proteins, composed of an extracellular ligand binding domain, an α-helical trans membrane segment and an intracellular tyrosine kinase domain. Ligand binding induces receptor dimerization, and auto-phosphorylation and activation of the intracellular kinase domain[96]. Of note, no natural ligand of HER2 has been identified. Nevertheless, HER2 can undergo dimerization with other ERBB receptors[97], and represents their preferred dimerization partner. The activated kinase domain that results then triggers downstream signaling pathways, such as MAPK, PI3K/AKT, protein kinase C and STAT, promoting cellular proliferation and inhibiting apoptosis[98]. Genetic alterations in HER2 can result in constitutive dimerization and activation of downstream signaling pathways, finally leading to uncontrolled cellular proliferation.

HER2 mutations is found in approximately 1%-3% of NSCLC, primarily in adenocarcinoma, in non-smokers and women[99]. Mutations usually consist of in frame insertions or point mutation in exon 20. On the contrary, HER2 amplification in NSCLC is not associated with benefit of anti-HER2 therapy[100].

Retrospective data indicate that HER2 targeted therapy present some degree of activity in HER2-mutated NSCLC. In a European cohort of 57 pre-treated HER2 mutated NSCLC patients receiving trastuzumab-based regimen, an ORR of 50% was reported, with a PFS of 5.1 mo[101]. In a prospective phase II trial, the antibody-drug conjugate ado-trastuzumab emtansine demonstrated an ORR of 44% in NSCLC patients harboring HER2 mutations, with a median PFS of 5 mo[102]. The most common treatment-related adverse events included grade 1 or 2 infusion reactions, thrombocytopenia, and elevated aspartate aminotransferase or alanine amino-transferase. No grade 4 or 5 adverse events were reported. More recently, the DESTINY-Lung01 trial evaluating the safety and efficacy of trastuzumab-deruxtecan, another antibody-drug conjugate targeting HER2, were presented at the ASCO meeting[103]. The DESTINY-Lung01 trial includes 2 cohorts: HER2 amplified (based on IHC) and HER2 mutated tumors[104]. Interim results concerning only the HER2 mutated population were reported. Of the 42 patients who received trastuzumab-deruxtecan, with a median of 2 prior treatment lines, the confirmed ORR was 61.9%, with a median duration of response not reached at data cut-off, and a median PFS estimation of 14 mo. However, toxicity was not negligible, as 64.3% of patients presented grade 3 or more adverse events (52.4 % drug-related). Of note, 11.9% of developed drug-related interstitial lung disease, all grade 2. Treatment related adverse events led to treatment interruption, dose reduction or treatment cessation in 59.5%, 38.1% and 23.8% of patients respectively. The randomized phase II DESTINY-Lung02 trial (NCT04644237) will compare a lower dose regimen of 5.4 mg/kg every 3-wk (Q3W) to the 6.4 mg/kg Q3W regimen used in DESTINY-Lung01 trial[105].

NEUREGULIN-1

The *neuregulin-1* (*NRG1*) gene is located on the long arm of chromosome 10 (10q23.1 region) and encodes a growth factor belonging to the complex family of proteins called heregulins, structurally related to the stimulation of ERBB receptors tyrosine kinase activity and EGF signals[106]. NRG1-receptor binding activates the ERBB2-ERBB3 heterocomplex and controls proliferation, differentiation, and survival in both normal and tumor cells through the predominant signaling cascades PI3K-AKT and MAP kinase[107].

NRG1 oncogenic gene rearrangements or fusions are rare, with an incidence of 0.2% on a wide retrospective molecular profiling that tested over 21'850 solid tumors[108]. They have been identified across a wide range of tumors including NSCLC (especially mucinous adenocarcinoma subtype), gallbladder cancer, pancreatic cancer, renal cell carcinoma, ovarian cancer and hepatic cholangiocarcinoma[109].

Detection of NRG1 gene fusions in solid tumors are made through RNA NGS[110]. *NRG1* aberrations appear to be mutually exclusive with oncogenic alterations *in EGFR, KRAS, ALK, ROS1*, and *rearranged during transfection* (*RET*).

NRG1 was first described in NSCC in 2014[110]. Invasive mucinous adenocarcinoma (IMA), representing approximately 5% of lung adenocarcinomas, are known to be more aggressive than more common types, such as acinar or papillary adenocarcinoma. *KRAS* mutations had been the only oncogenic driver commonly detected in IMAs (in 50%–80% of cases), but *CD74-NRG1* fusions are now detected in 14.7% of *KRAS* negative IMAs. NRG1 positivity confers worse outcomes in lung adenocarcinoma in retrospective data[111]. Given the potential therapeutic implications of this genetic alteration, the interest in evaluating the prevalence of NRG1 fusions has increased over the last five years.

The use of afatinib (tyrosine kinase inhibitor, targeting ERBB) showed interesting results in case reports for the treatment of *NRG1* fusion-positive in cancers of lung (*SDC4-, SLC3A2-* and *CD74-NRG1* gene fusion) and hepatocellular (*ATP1B1-NRG1* gene fusion) origin[112]. Further studies are ongoing.

RET

The *RET* gene, located on the chromosome 10 (10q11.2), encodes a tyrosine kinase receptor located to the cell surface. Its intracellular kinase domain shares 37% homology with the ALK kinase domain[113]. RET is a receptor for Glial Cell Line-Derived neurotrophic factor (GDNF), a family extracellular signaling molecules notably involved in neuronal development[114]. The binding of GDNF to a co-receptor GDRalpha and then to RET, leads to a RET-dimerization and further autophosphorylation of the kinase domains. This activates the intracellular signal transduction process, notably the RAS, MAPK/ERK, PI3K/AKT and JAK/STAT pathways[113]. RET is normally involved in enteric nervous system and urogenital tract development. RET loss of function is associated with Hirschprung disease[115], and activating mutations to MEN-2 syndrome[116]. Rearrangements of the *RET* gene (*e.g.,* the apposition of the C-terminal region of RET protein with the N-terminal region of another protein) can induce a constitutive activation of the RET kinase.

Rearrangements in RET with various partners have been described, the most common in NSCLC being KIF5B and CCDC6[117]. They have been identified in 1%-2% of lung adenocarcinomas, more often in never smoker and younger patients[113]. IHC is probably unreliable for the detection of RET-rearrangements, and FISH or NGS are preferred[113].

The phase I/II LIBRETTO 001 trial[118] evaluated selpercatinib in NSCLC patients. It showed an ORR of 64% in 105 patients previously treated with platinum-based chemotherapy and 85% in previously untreated patient (39 patients). Median duration of response was 17.5 mo in previously treated patients. Interestingly, among the 11 patients with central nervous system disease, 91% had an intracranial response.

Following these results, selpercatinib was granted accelerated approval by the FDA[119]. Selpercatinib is currently being evaluated in phase III, *vs* platinum-based chemotherapy +/- pembrolizumab, in the LIBRETTO-431 trial[120].

Pralsetinib (BLU-667), another selective RET inhibitor has also been granted accelerated approval from the FDA based on the results of the ARROW-study (NCT03037385), a basket trial in which patients with RET-fusions positive cancer received pralsetinib 400mg orally once daily. Among 89 patients with RET-fusion positive NSCLC pretreated with platinum-based chemotherapy, ORR was 57%, with 80% of ongoing responses at 6 mo[121]. Among 27 patients with previously untreated NSCLC, the ORR was 70% with 58% of response ongoing at 6 mo. Praseltinib is currently being compared to the standard treatment approach of platinum-based chemotherapy ± pembrolizumab in RET-fusion positive NSCLC in the AcceleRET trial (NCT04222972)[122].

Other non-RET-selective TKIs have shown some activity in RET-fusion positive NSCLC: Cabozantinib for example displayed an ORR of 28% in a single-center, phase II trial, of patients with RET-rearranged NSCLC, of whom 75% had received prior chemotherapy[123]. In an international registry of patients with RET-rearranged

NSCLCs, cabozantinib, vandetanib, and sunitinib had rather limited activity, with overall response rates of 37%, 18%, and 22%, respectively[124].

Different mechanisms of RET-inhibition resistance have been described including the emergence of solvent front mutation in the *RET*-gene (*e.g.* in the RET G810 residue, in the kinase solvent front)[125] as well as acquired MET or KRAS amplifications[126].

CONCLUSION

Driver mutations have significantly altered the diagnostic work-up and reshaped the oncology treatment paradigm. Recently, several new driver mutations have been identified in metastatic NSCLC, with some leading to therapeutic success and others, failure. We have summarized the current landscape of actionable oncogenic alterations and their therapies in Table 1. A better understanding of the biology of various subtypes of each driver mutation will help not only to match the optimal treatment to each patient, but also elucidate their respective resistance mechanisms, allowing for greater precision medicine. Many trials are ongoing, some of them through serial tumor or plasma biopsies and multiplex molecular testing. The optimal targeted therapy sequence for each driver mutation is yet to be fully determined. Furthermore, it is unclear whether a multi-kinase inhibitor or highly selective therapy is the best choice for some alterations, though the latter tend to have more favourable toxicity profiles.

Given the portfolio of possible mutations and targeted therapies to offer, multiplex NGS testing should be standard practice. Barring a therapeutic emergency, no patient should be started on systemic therapy before a comprehensive molecular analysis has been completed. In the twentieth century, every gene matters, and it would be unethical to deny patients access to proven targeted therapies given the efficacy and favourable toxicity profile of such drugs.

Table 1 Selected oncogenic drivers and their treatments in non-small cell lung cancer

	Targeted therapy	ORR	PFS	OS
EGFR	Erlotinib[1][40]	62%-83%	9.7-13.1 mo	19.3-26.3 mo
	Gefitinib[1][4]	73.7%	9.5 mo	22 mo
	Afatinib[1][5]	56%-67%	11.1 mo	25.8 mo
	Dacomitinib[1][127]	74.9%	14.7 mo	34 mo
	Osimertinib[1][128]	80%	18.9 mo	38.6 mo
ALK	Crizotinib[1][53]	75.5%	11 mo	57 mo
	Ceritinib[1][129]	72.5%	16.6 mo	NA
	Brigatinib0[130]	79%	24 mo	NA
	Alectinib[1][131]	82.9%	35 mo	NA
	Lorlatinib[1][54]	76%	NA	NA
ROS1	Crizotinib[1][132]	63-72%	15.9-19.2 mo	51 mo
	Lorlatinib[1][133]	62%	19.3 mo	NA
	Entrectinib[1][134]	77%	19 mo	NA
BRAF	Dabrafenib-trametinib[1][135]	64%	10.9 mo	24.6 mo
MET	Crizotinib[50]	32%	7.3 mo	NA
	Cabozantinib[136]	NA	NA	NA
	Capmatinib[1][137]	68%	9.7 mo	NA
	Tepotinib[138]	46%	NA	NA
NTRK	Entrectinib[1][139]	70%	NA	NA
	Larotrectinib[1][140]	75%	NA	NA
RET	Selpercatinib[1][141]	85%	NA	NA
	Pralsetinib[1][121]	70%	NA	NA
KRAS	Sotorasib[142]	32.2%	10.2 mo	NA
	Adagrasib[143]	45%	NA	NA
HER2	Trastuzumab-deruxtecan[144]	62%	14 mo	NA
NRG1	Afatinib[112]	NA	NA	NA

[1]Food and Drug Administration approved. ORR: Objective response rate; PFS: Progression-free survival; OS: Overall survival; EGFR: Epidermal growth factor receptor; ALK: Anaplastic lymphoma kinase; ROS1: c-ROS oncogene 1; NTRK: Neurotrophic receptor tyrosine kinase; RET: Rearranged during transfection; KRAS: Kirsten rat sarcoma; HER2: Human epidermal growth factor 2; NRG1: Neuregulin-1; BRAF: V-raf murine sarcoma viral oncogene homolog B. NA: Not available.

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ORIGINAL ARTICLE

Basic Study

Cytotoxic CD8+ T cells and tissue resident memory cells in colorectal cancer based on microsatellite instability and BRAF status

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Abstract

BACKGROUND

Recent studies in non-colorectal malignancy have associated T resident memory (T_{RM}) cells with improved patient survival. It is unknown if T_{RM} plays a role in colorectal cancer (CRC).

AIM

To examine the potential role of T_{RM} cells in providing immunogenicity in CRC stratified by microsatellite instability (MSI) and BRAF status.

METHODS

Patients with known MSI and BRAF mutation status were eligible for inclusion in this study. CRC tumour sections stained with haematoxylin and eosin were

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microscopically reviewed and the images scanned prior to assessment for location of invading edge and core of tumour. Sequential sections were prepared for quantitative multiplex immunohistochemistry (IHC) staining. Opal Multiplex IHC staining was performed with appropriate positive and negative controls and imaged using a standard fluorescent microscope fitted with a spectral scanning camera (Mantra) in conjunction with Mantra snap software. Images were unmixed and annotated in inForm 2.2.0. Statistical analysis was performed using Graphpad Prism Version 7 and Stata Version 15.

RESULTS
Seventy-two patients with known MSI and BRAF status were included in the study. All patients were assessed for MSI by IHC and high resolution capillary electrophoresis testing and 44 of these patients successfully underwent quantitative multiplex IHC staining. Overall, there was a statistically significant increase in CD8+ T_{RM} cells in the MSI (BRAF mutant and wild type) group over the microsatellite stable (MSS) group. There was a statistically significant difference in CD8+ T_{RM} between high level MSI (MSI-H):BRAF mutant [22.57, 95% confidence interval (CI): 14.31-30.84] *vs* MSS [8.031 (95%CI: 4.698-11.36)], P = 0.0076 andMSI-H:BRAF wild type [16.18 (95%CI: 10.44-21.93)] *vs* MSS [8.031 (95%CI: 4.698-11.36)], P = 0.0279. There was no statistically significant difference in CD8 T cells (both CD8+CD103- and CD8+CD103+T_{RM}) between MSI-H: BRAF mutant and wild type CRC.

CONCLUSION
This study has shown that CD8+ T_{RM} are found in greater abundance in MSI-H CRC, both BRAF mutant and MSI-H:BRAF wild type, when compared with their MSS counterpart. CD8+ T_{RM} may play a role in the immunogenicity in MSI-H CRC (BRAF mutant and BRAF wild type). Further studies should focus on the potential immunogenic qualities of T_{RM} cells and investigate potential immunotherapeutic approaches to improve treatment and survival associated with CRC.

Key Words: Tissue resident memory cells; Resident memory T cells; Colorectal cancer; Microsatellite instability; BRAF; DNA mismatch repair; Immunotherapy; Prognosis

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Core Tip: Prior to this study, whether T resident memory (T_{RM}) cells exist in colorectal cancer (CRC) was poorly understood. This study has identified and characterize T_{RM} cells within human CRC. CD8+ TRM cells are found in greater abundance in both high microsatellite instability (MSI-H) BRAF mutant and MSI-H:BRAF wild type CRC when compared with their microsatellite stable counterpart. CD8+ TRM may play a role in the immunogenicity in both BRAF mutant and BRAF wild type MSI-H CRC. Further studies should focus on the potential immunogenic qualities of TRM cells and investigate potential immunotherapeutic approaches to improve treatment and survival associated with CRC based on TRM.

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INTRODUCTION

Colorectal cancer (CRC) may be divided into microsatellite stable (MSS) and high microsatellite unstable (MSI-H) CRC. Approximately 15% of CRC are MSI-H and are more likely to be immunogenic with abundant tumour infiltrating lymphocytes (TILs)[1-3]. On the other hand, MSS CRC are less likely to be immunogenic, with

sparse/limited TILs. Abundance of TILs has been associated improved survival[4-14] and is widely believed to provide MSI-H CRC with a favourable prognosis[15,16] despite the higher grade features also associated with MSI-H CRC, such as larger diameter, poorly differentiated, mucinous tumours.

However, while it is widely known that MSI-H CRC is associated with TILs, there is significant debate as to the composition of TILs within CRC and which component of TILs is most important. Various reports have suggested that within TILs, CD8+, CD4+, CD3+, FOXP3+ and CD45RO+ regulatory cells have important roles in immunosurveillance. There has been no consensus in recent studies. Nosho *et al*[17] reported on the benefits of CD45RO+ cells, Guidoboni *et al*[18] on cytotoxic CD3+ and CD8+ cells, Salama *et al*[19] reported that FOXP3+ cells were associated with favourable prognosis respectively. Potentially, the interaction of all of these cells may provide benefit as these cells have different profiles and roles within an intricate orchestrated immune response. It has been believed for decades that T cells are recruited in response to cancer, and constantly recirculate in the blood and lymphatic system. The benefit of these TILs in CRC has been reported and has been subject to recent reviews[20,21].

More recently a new lineage of T cells has been discovered which is believed to provide immunity to solid tumours[22-24]. Tissue resident memory (T_{RM}) cells, particularly CD8+ $T_{RM,}$ have been identified in non-colorectal malignancies and its presence has already been shown to be associated with better survival outcomes in lung cancer[25], ovarian cancer[26], breast cancer[27-29] and melanoma[24,30], particularly CD8+ T_{RM}[24-27,31-36]. The difference between CD8+ T_{RM} and conventional cytotoxic CD8+ T cells is that T_{RM} cells, by definition, do not recirculate—rather they remain in peripheral tissue. This is because T_{RM} cells are not just a continuous circulation of T cells, but cells committed to their tissue of residence including tumour tissue, thereby providing immediate and long-term protection against invaders. T_{RM} cells are believed to produce interferon-gamma, granzyme B and perforin[30].

T_{RM} cells at epithelial surfaces develop from precursor cells through a process that relies on transcription factors and cytokines[37] and can be identified by the expression of CD103 (and CD69). Both CD103 and CD69 contribute to the retention of these cells within epithelia and prevent these cells from exiting their resident tissue. CD103 is the alpha chain of integrin, which binds to E-Cadherin which is an epithelial adhesion molecule. Furthermore, T_{RM} cells express checkpoint receptors such as programmed death 1 (PD-1), CTLA4 and Tim-3, potential targets for current immunotherapy strategies. If present in CRC, T_{RM} may represent an avenue for improving survival in CRC.

In this study, we have chosen CD103 status to identify T_{RM} cells. CD39, CD69 and CD103 have been associated with T_{RM}[36,37], with CD103 reported to strongly correlate with a favourable prognosis in cancer patients[38]. While there are studies which have suggested the importance of CD4+ T_{RM} cells[39], the majority of studies have associated improved survival with CD8+ T_{RM} cells[24-26,31-36] and for this reason the main outcome measure chosen for this study was the abundance of CD8+ T_{RM} in the CRC tumour microenvironment. Secondary outcomes included assessing the presence of CD8+ T_{RM} and cytotoxic CD8+ non-TRM cells. Results of the above outcome measures were pooled by microsatellite instability (MSI) and BRAF status.

MSI status was chosen because it is a biomarker widely considered to influence immunogenicity. BRAF status was also chosen because in CRC it has been shown that presence of BRAFV600E mutation nearly always excludes Lynch syndrome and absence of BRAF mutation (BRAF wild type) may be associated with Lynch syndrome (although 30%-40% of MSI-H:BRAF wild type may be sporadic)[40]. Thus this study examined the potential role of T_{RM} cells in providing immunogenicity in CRC stratified by MSI and BRAF status, with CRC divided into three groups: MSS, MSI-H:BRAF mutant (representing sporadic MSI-H, usually associated with epigenetic silencing of MLH-1) and MSI-H:BRAF wild type (approximately 60%-70% associated with Lynch syndrome)[40].

MATERIALS AND METHODS

Patient

Seventy-two patients with known MSI and BRAF status confirmed by immunohistochemistry (IHC) and high resolution capillary electrophoresis were eligible for inclusion in this study. These patients also had detailed clinical, operative, pathological, adjuvant treatment and follow-up data from a cancer database which

was analyzed retrospectively. Institutional board approval was obtained, and patients had provided written consent for the use of their information for research (CH62/62011-136 HREC/11/CRGH206).

Histopathology

Histopathology slides prepared with haematoxylin and eosin were reviewed using an Olympus BX53 microscope to find a representative slide which shows the tumour invading edge and core. These slides were then scanned electronically and the scanned images were reexamined by a pathologist to mark out the invading edge and core. From these blocks, representative slides were recut using a microtome in preparation for quantitative multiplex IHC staining.

Of the 72 original patient tumour specimens prepared on representative slides, 44 formalin fixed paraffin embedded (FFPE) tumour sections were successfully stained with quantitative multiplex IHC staining. All IHC staining was performed on 4-μm-thick sections and stained using an Opal Multiplex IHC assay kit (PerkinElmer, Waltham, MA, United States) and optimised in-house. Briefly, FFPE tissue sections were deparaffinized and rehydrated prior to antigen retrieval by boiling in basic (10 mmol/L Tris base, 1 mmol/L EDTA, 0.05% Tween 20, pH 9.0) buffer. The sections were then incubated with 3% hydrogen peroxide for 20 min at room temperature before washing and blocking with Perkin Elmer Antibody diluent/Block buffer. Sections were then incubated with a single unconjugated primary antibody for 35 min, washed and then incubated with Opal Polymer HRP (Akoya Biosciences, United States) for 10 min. After washing, sections were incubated with Opal fluorochromes at a 1:100 dilution made up in tyramide signal amplification reagent (PerkinElmer, Waltham, MA, United States) for 10 min. The antigen retrieval step was repeated, and tissue was stained for subsequent antibodies as described above. Finally, sections were stained with DAPI (cell signalling technologies) for 5 min and then mounted using Prolong Diamond (Life Technologies). Slides were stored in the dark until imaging was complete. The following antibodies were used; CD8 (ab4055 clone; Abcam), CD103 [EPR4116(2) clone; Abcam] PD1 (NAT105 clone, Cell Marque). The Mantra imaging platform (PerkinElmer) was used for imaging in combination with Mantra snap software for data acquisition. For all quantitative analysis up to 10 randomly selected regions of interest per section were analyzed. InForm advanced image analysis software (PerkinElmer, MA, United States) (PerkinElmer) was used to process and analyze images, with each tissue manually segmented into tissue regions.

Statistical analysis

Statistical analysis was performed using Graphpad Prism Version 7 and Stata Version 15.

RESULTS

T_{RM} T cells are significantly increased in tumours with defective DNA mismatch repair

Seventy-two patients with known MSI and BRAF status met the eligibility criteria for this study, and of these 44 patient tumour specimens were successfully stained by quantitative multiplex IHC staining (Figure 1). The percentage and densities of total CD8+ T cells, T_{RM} T cells, and non-T_{RM} CD8+ T cells were calculated for MSS, MSI-H:BRAF mutant and MSI-H:BRAF wild type subgroups. An unpaired student *t* test was used to compare the mean % T_{RM}/non-T_{RM} cells/total cells between the three groups for each outcome measure. Where there were significant outliers, the median was used instead of mean, and a Mann Whitney *U* test was performed to compare the median between the three groups. This showed that compared to healthy control all three groups had a statistically greater abundance of CD8+ T cells (Figure 1).

There was a significant difference in the proportion of CD8+ T cells between MSI-H:BRAF mutant and MSS subgroups with 17.24% [95% confidence interval (CI): 9.334-25.15] of nucleated cells in MSS compared to 28.14% (95%CI: 20.38-35.89) in MSI-H:BRAF mutant and 22.82 (95%CI: 16.02-29.62) in MSI-H:BRAF wild type subgroups. On Mann Whitney *U* analysis, there was a statistically significant difference between MSI-H:BRAF mutant and MSS subgroups (P = 0.0417). Although there was a greater percentage of CD8+ T cells in the MSI-H:BRAF wild type subgroup when compared to MSS, this did not reach statistical significance [22.82 (95%CI: 16.02-29.62) *vs* 17% (95%CI: 9.334-25.15), P = 0.2841].

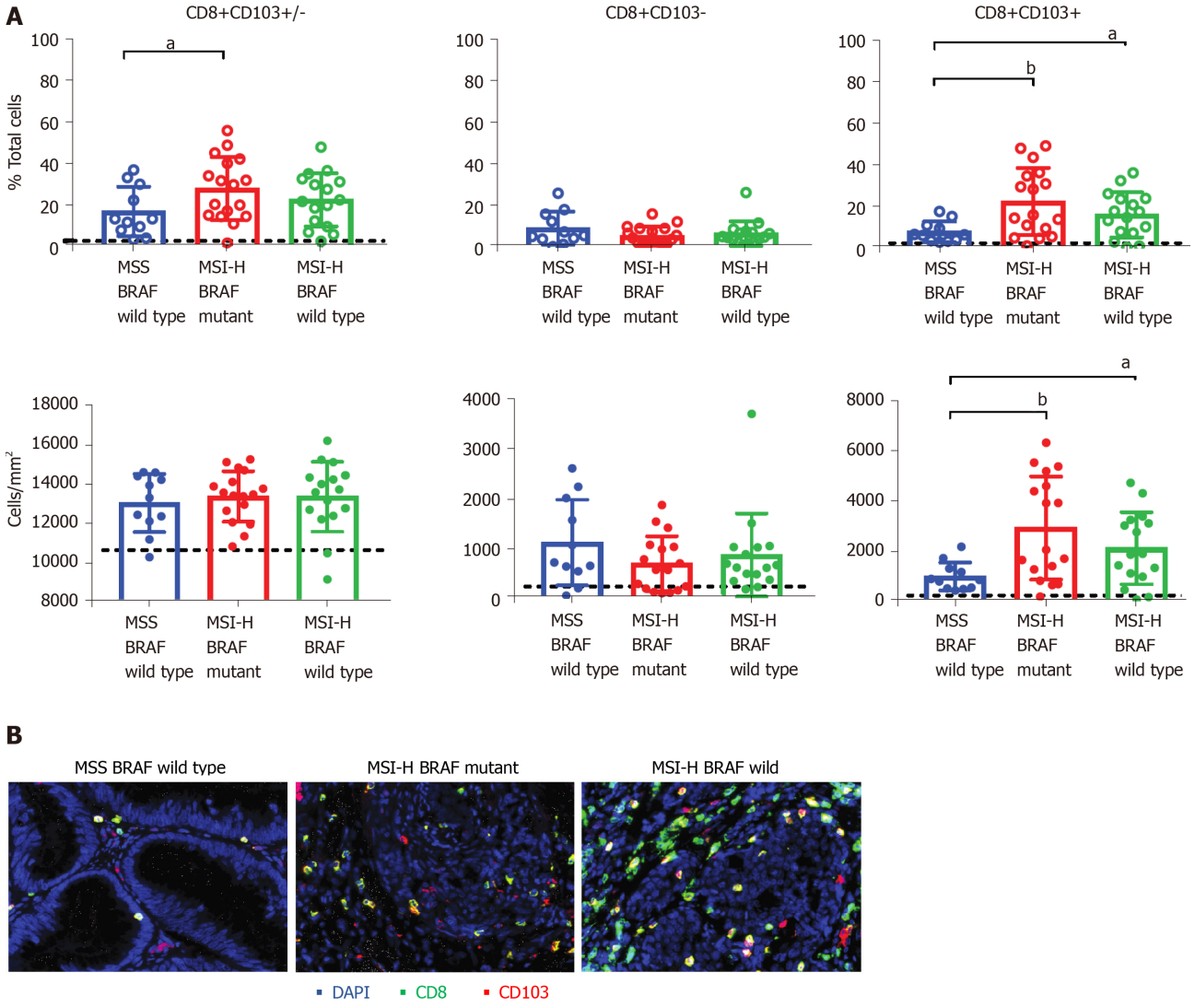


Figure 1 Tissue resident memory T cells are significantly increased in microsatellite instability high tumours. A: Quantitative immunohistochemistry staining of tumour specimen show the proportion (top panels) and densities (bottom panels) of total CD8+ T cells (left panels), CD103-CD8+ (non-T_{RM}) T cells (middle panels) and CD103+CD8+ resident memory (T_{RM}) T cells (right panels) in CRC tumours with microsatellite stable BRAF wildtype and microsatellite instability (MSI) high BRAF mutants and MSI high BRAF wildtype groups. Figure shows the mean and SD of n = 11-17 samples and the statistical differences were calculated using unpaired and non-parametric Mann-Whitney test T test ($^aP < 0.05$; $^bP < 0.001$). Black line shows the levels determined from normal healthy colorectal tissues (n = 1); B: Representative tissue staining shows the staining for CD8 (green) and CD103 (red) and DAPI (blue) in the three groups of patients. MSS: Microsatellite stable; MSI-H: High microsatellite instability.

Importantly, there were significant differences in the proportions of CD8+ T_{RM} cells between the three subgroups of CRC. Compared to MSS, the MSI-H:BRAF mutant subgroup had a significantly higher proportion of CD8+ T_{RM} cells (Figure 1) with 22.5% (95%CI: 14.31-30.84) in MSI-H:BRAF mutant subgroup and 8.0% in MSS subgroup (95%CI: 4.698-11.36, P = 0.0076). Similarly, the MSI-H:BRAF wild type subgroup had significantly higher CD8+ T_{RM} (16.18%, 95%CI: 10.44-21.93) when compared to MSS (8%, 95%CI: 4.698-11.36 and P = 0.0279). The difference in abundance of T_{RM} between MSI-H: BRAF mutant and MSI-H:BRAF wild type subgroups was not significant (Figure 1). There was no statistically significant difference in non-T_{RM} CD8+ cells between the three subgroups.

PD-1 expression on CD8+ T cells is not impacted by MSI status

We next sought to determine whether the expression of checkpoint receptor, PD-1 was impacted by MSI status or BRAF mutation. We enumerated the proportion of T cells expressing PD-1 in all three subgroups. PD-1 expression was present in both T_{RM} T cells and non-T_{RM} T cell populations, although the proportion of non-T_{RM} T cells expressing PD-1 was slightly higher than that of the T_{RM} T cell population (Figure 2). While there was a trend to increased PD-1+ expression in the T_{RM} T cell population in MSI-H:BRAF wild type compared to MSI-H:BRAF mutant and MSS subgroups, there

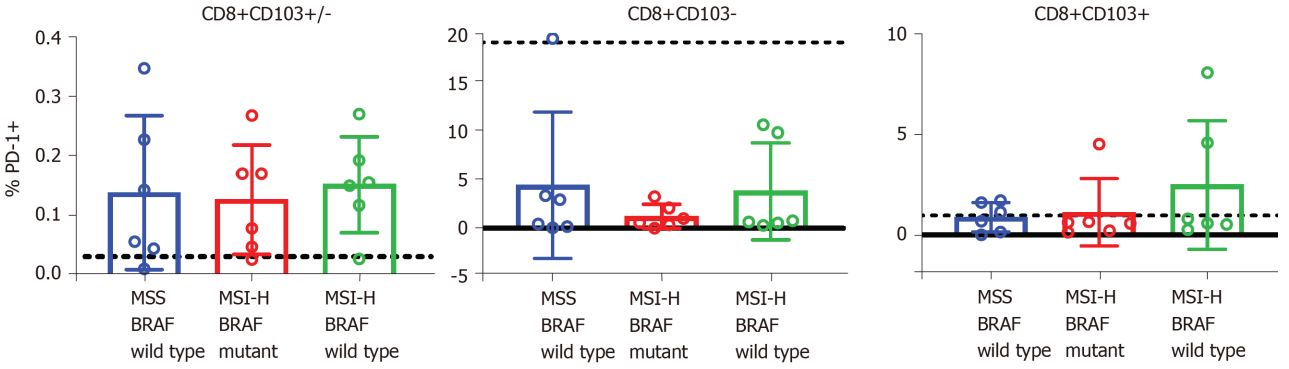


Figure 2 Programmed cell death protein-1 expression on CD8+ T cells is not impacted by microsatellite instability or BRAF status. Quantitative immunohistochemistry staining was used to determine the expression of programmed cell death protein-1 (PD-1) on CD8+ T cell populations. Figure shows the proportion of total CD8+ T cells (CD8+CD103+/-) (left panel), CD103-CD8+ (non-T_{RM}) T cells (middle panel) and CD103+CD8+ T_{RM} cells (right panel) expressing PD-1. Figure shows the mean and SD for 5-6 samples/group. PD-1: Programmed death 1; MSS: Microsatellite stable; MSI-H: High microsatellite instability.

was, however, no statistically significant differences in the proportion of T cells expressing PD-1 between the three groups.

DISCUSSION

Characteristics of TILS within CRC have been poorly defined to date, and there is a paucity of information on T_{RM} T cells in CRC. In humans, within the circulation, two types of memory T cells exist — central memory (TCM) and effector memory (TEM). For decades, it was thought that T lymphocyte populations in peripheral tissues including TILs were largely maintained by the continuous circulation of these memory T cells as well as other circulatory cells. Recent studies have challenged this concept and provided evidence for the existence of another subset of $CD8^+$ T cells — tissue-T_{RM} T cells. These cells are permanently resident (*i.e.*, do not recirculate) in both lymphoid and non-lymphoid tissues and represent a distinct subset to circulatory TEM or TCM.

Prior to this study the existence of T_{RM} cells in CRC was poorly understood and the aim of this study was to identify and characterize T_{RM} cells within human CRC. If present, T_{RM} cells may be important in the protection against recurrence, dissemination and metastasis. A favourable prognosis associated with the presence of T_{RM} cells has already been reported in studies of high-grade serous ovarian cancer patients, non-small cell lung carcinoma patients and breast cancer[41]. In lung cancer, although only a small proportion of CD8+ T cells were reported to be T_{RM}, the majority of T_{RM} cells were tumour-reactive. Studies in melanoma have also shown an association between T_{RM} and favourable prognosis[30,42].

This present study showed that T_{RM} cells exist in MSS, MSI-H:BRAF mutant and MSI-H:BRAF wild type CRC. However, they are in greater abundance in MSI-H than MSS CRC. This is supported by the findings of a recent study by de Vries *et al*[21] on 35 CRC tissues which demonstrated that CD103+ T_{RM} cells were most abundant in MSI-H CRC. In that study 13 MSI-H CRC were included and BRAF status was not considered. Our study had 42 MSI-H CRC patients which were all subject to immunoscoring, of which 33 went on to have multiplex IHC staining to look for presence of T_{RM} cells and of these 17 were BRAF mutant and 16 were BRAF wild type. Of the 30 MSS CRC patients, 11 went on to have multiplex IHC staining to look for presence of T_{RM} cells. Our study showed that both MSI-H:BRAF mutant and BRAF wild type subgroups had significantly greater abundance of T_{RM} cells when compared to the MSS subgroup. There was no statistically significant difference between MSI-H:BRAF mutant and BRAF wild type subgroups.

There was a trend to increased PD-1+ in the T_{RM} T cell population in MSI-H:BRAF wild type compared to MSI-H:BRAF mutant and MSS subgroups, but this was not statistically significant. This is an important finding as in advanced CRC this T cell subpopulation amongst other PD-1+ immune cells may be exhausted by PD-1/PD-1 ligand binding, a mechanism of escaping immunosurveillance by tumours. Reactivation of exhausted PD-1+ T_{RM} cells may provide therapeutic opportunities that may be exploited in MSI-H:BRAF wild type CRC. PD-1 expression has been reported to be more prominent in cells with a T_{RM} phenotype[25,43], and in the case of CRC it may

be their numbers that impacts the outcome.

The potential ways which CD8+ T_{RM} cells can be harnessed to improve the success of immunotherapeutic targets has already been the focus of several general reviews[38,44-46] as well as in breast cancer[31] and melanoma[47,48], with Edwards *et al*[42] reporting that CD8+ T_{RM} cells being prognostic in metastatic melanoma and potentially initiating response to immunotherapy with anti-PD-1. Therefore, the role of T_{RM} cells could have important implications for checkpoint-inhibition therapy in CRC[49].

The mechanisms by which T_{RM} cells confer immunity remains unclear. Recent studies by Park *et al*[24] have demonstrated that depletion of T_{RM} cells in melanoma triggered tumour outgrowth of mice with occult melanoma, demonstrating that T_{RM} cells have an important role in cancer surveillance and equilibrium and may keep cancer cells dormant[50]. This effect may be long term as T_{RM} cell populations are able to persist in barrier tissues for a prolonged period of time[51]. Menares *et al*[52] suggest that the mechanism by which T_{RM} cells provide immunity is *via* cross-talk with dendritic cells to trigger the spread of cytotoxic CD8+ T cells in response to tumour.

Limitations

The use of a single marker (CD103) to define T_{RM} cells is a limitation. However, many studies have consistently shown that CD103+CD8+ T cells have the hallmark of T_{RM} T cells[24,53]. Further, the study sample size was small (n = 72) and we were only able to successfully perform quantitative multiplex IHC staining and imaging on 44 samples. Nonetheless, this is to date the largest study on T_{RM} cells in CRC.

CONCLUSION

This study has shown that in CRC a significant fraction of TILs is made up of T_{RM} cells and are found in greater abundance in MSI-H:BRAF mutant and MSI-H:BRAF wild type compared with MSS CRC tumours. Acknowledging that there is a complex interaction of factors at play in influencing the prognosis in CRC, the abundance of T_{RM} cells may contribute to the favourable prognosis observed in MSI-H CRC when compared to MSS[15,16]. This is because MSS CRC has significantly less T_{RM} cells when compared to MSI-H CRC. The abundance of T_{RM} cells in MSI-H CRC may also explain the reduced likelihood of metastases in patients with MSI-H CRC[54] and also the difference in response to immunotherapy between MSI-H CRC and MSS CRC[20]. It is important to understand that while T_{RM} cells can provide immunity in solid organ tumours, the immunity conferred is by no means absolute and cancers still arise and continue to disseminate despite the presence of T_{RM} cells. Further studies should focus on the potential immunogenic qualities of T_{RM} cells and investigate potential immunotherapeutic approaches to improve treatment and survival associated with CRC.

ARTICLE HIGHLIGHTS

Research background

The presence of resident memory T (T_{RM}) cells has already been reported in studies of high-grade serous ovarian cancer patients, non-small cell lung carcinoma patients and breast cancer, but there has been limited evidence on T_{RM} cells in the scientific literature in colorectal cancer (CRC). This is a landmark study on T_{RM} cells in CRC.

Research motivation

With T_{RM} cells showing promise in several solid organ tumour studies, the premise of this study was to evaluate if T_{RM} cells are present in CRC and thus potentially be a target for immunotherapy/novel target therapy in the future.

Research objectives

The objective of this study was to examine the potential role of T_{RM} cells in providing immunogenicity in CRC stratified by microsatellite instability (MSI) and BRAF status.

Research methods

Formalin fixed paraffin embedded tumour sections were successfully stained using quantitative multiplex IHC staining. All IHC staining was performed using an Opal Multiplex immunohistochemistry (IHC) assay kit (PerkinElmer, Waltham, MA, United

States) and optimised in-house.

Research results

This study has shown that CD8+ T_{RM} are found in greater abundance in both high level MSI (MSI-H):BRAF mutant and MSI-H:BRAF wild type CRC when compared with their microsatellite stable (MSS) counterpart. CD8+ T_{RM} may play a role in the immuno-genicity in both BRAF mutant and BRAF wild type MSI-H CRC. The abundance of T_{RM} cells may contribute to the favourable prognosis observed in MSI-H CRC when compared to MSS CRC.

Research conclusions

T_{RM} cells are found in greater abundance and contributes to the immunogenicity of MSI-H CRCs.

Research perspectives

T_{RM} cells may be a target for immunotherapy/novel target therapy in MSI-H CRCs, and future research should focus on the potential value of T_{RM} cells, as well as therapeutic agents that may stimulate T_{RM} activity or adoptive cell transfer aimed at harvesting and utilizing the patients' own immune cells such as specially altered T-cells to precisely and specifically target cancer cells.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Positron emission tomography complete metabolic response as a favorable prog-nostic predictor in esophageal cancer following neoadjuvant chemotherapy with docetaxel/cis-platin/5-fluorouracil

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Abstract

BACKGROUND

^{18}F-fluorodeoxyglucose-positron emission tomography (PET)/computed tomography is useful in diagnosing lymph node and distant metastases of esophageal cancer. However, its value for predicting survival is controversial.

AIM

To evaluate the value of PET complete metabolic response (CMR) as a prognostic predictor for esophageal cancer.

METHODS

Between June 2013 and December 2017, 58 patients with squamous cell esophageal cancer who underwent neoadjuvant chemotherapy (NAC) in Oita University were enrolled in this retrospective cohort study. Tumors were clinically staged using fluorodeoxyglucose-PET/computed tomography before and after NAC. After NAC, maximal standardized uptake value ≤ 2.5 was defined as PET-CMR, and maximal standardized uptake value > 2.5 was defined as non-PET-CMR. We compared short-term outcomes between the PET-CMR group and non-PET-CMR group and evaluated prognostic factors by univariate and multivariate analyses.

RESULTS

The PET-CMR group included 22 patients, and the non-PET-CMR group included 36 patients. There were no significant differences in intraoperative and post-

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operative complications between the two groups. Five-year relapse-free survival and overall survival in the PET-CMR group were significantly more favorable than those in the non-PET-CMR group (38.6 mo *vs* 20.8 mo, $P = 0.021$; 42.8 mo *vs* 25.1 mo, $P = 0.011$, respectively). PET-CMR was a significant prognostic factor in terms of relapse-free survival by univariate analysis (hazard ratio: 2.523; 95% confidence interval: 1.034–7.063; $P < 0.041$). Particularly, PET-computed tomography negative N was an independent prognostic factor of relapse-free survival and overall survival by multivariate analysis.

CONCLUSION

PET-CMR after NAC is considered a favorable prognostic factor for esophageal cancer. Evaluation by PET-computed tomography could be useful in clinical decision making for esophageal cancer.

Key Words: Esophageal cancer; Neoadjuvant chemotherapy; Positron emission tomography/computed tomography; Complete metabolic response; Prognostic factor; Docetaxel, cisplatin plus 5-fluorouracil

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Core Tip: The study aimed to evaluate the value of positron emission tomography (PET)/computed tomography complete metabolic response (CMR) as a prognostic predictor for esophageal cancer. Fifty-eight patients with esophageal cancer who underwent neoadjuvant chemotherapy were enrolled. The PET-CMR group included 22 patients, and the non-PET-CMR group included 36 patients. Five-year relapse-free survival and overall survival in the PET-CMR group were significantly more favorable than those in the non-PET-CMR group. PET-CMR was a significant prognostic factor in terms of relapse-free survival by univariate analysis. PET-CMR after neoadjuvant chemotherapy is considered a favorable prognostic factor for relapse-free survival in patients with esophageal cancer.

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INTRODUCTION

Esophageal cancer is the eighth most common type of cancer worldwide, with a 5-yr overall survival (OS) of 35% after primary esophagectomy[1]. Neoadjuvant chemotherapy/radiotherapy (CRT) has been introduced to improve survival of patients with advanced esophageal cancer[2,3]. CRT followed by esophagectomy is considered a standard strategy for locally advanced esophageal cancer in Western countries[3,4]. In contrast, neoadjuvant chemotherapy (NAC) with cisplatin plus 5-fluorouracil (CF) followed by esophagectomy is the standard strategy according to the JCOG 9907 trial in Japan[5].

With the expectation of further anticancer effect, triplet NAC with docetaxel, cisplatin plus 5-fluorouracil (DCF) was introduced for locally advanced esophageal cancer, and anticancer effects and survival benefits of DCF were reported in previous studies[6,7]. Furthermore, to clarify the feasibility and effectiveness of DCF and DCF/radiotherapy compared to CF as neoadjuvant therapy for locally advanced esophageal cancer, a randomized controlled study is underway in Japan (JCOG1109)[8].

Proper evaluation of preoperative chemotherapy response and lymph node (LN) metastasis before surgery is important for predicting prognosis in patients who have received neoadjuvant therapy[9,10]. Computed tomography (CT) is widely used to determine staging in esophageal cancer. However, the accuracy of CT in detecting regional LN metastasis in esophageal cancer is still unsatisfactory[11]. Recent studies

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have shown that ^{18}F-fluorodeoxyglucose (FDG)-positron emission tomography (PET) can determine the degree of metabolic activity in tumor cells and can improve tumor staging for patients with esophageal cancer[12,13]. Furthermore, several studies have shown that FDG-PET/CT is useful for diagnosing metastatic LNs and distant metastasis and for detecting the recurrence of esophageal cancer after surgery[12-14]. However, the value of FDG-PET/CT to predict survival in patients with esophageal cancer is controversial. In this study, we aimed to evaluate the value of PET complete metabolic response (CMR) in esophageal cancer following NAC as a prognostic predictor.

MATERIALS AND METHODS

Patients

We reviewed data from 70 consecutive patients with squamous cell esophageal cancer who were preoperatively evaluated with FDG-PET/CT before and after NAC with DCF between June 2013 and December 2017 in Oita University. Those who received definitive radio/chemotherapy after NAC (n = 11) and underwent esophagectomy in another hospital (n = 1) were excluded. Thus, 58 patients received esophagectomy after NAC and were enrolled in this retrospective study. NAC was recommended for patients with resectable clinical stage T2-T4 esophageal cancer and for those with tumors and clinical LN metastases or resectable supraclavicular LN metastasis (clinical M1 3) according to the TNM Classification of Malignant Tumors, 7th edition[15]. Indications for NAC with DCF in our institution are age < 80-years-old and performance status 0 or 1. Patients with a history of adverse events from docetaxel were excluded from receiving NAC with DCF. The clinicopathologic profiles of the tumors were based on the TNM Classification of Malignant Tumors, 7th edition[15]. This study was approved by the institutional Ethical Review Board of Oita University Faculty of Medicine (Approval No. 1602).

Schedule of treatment

The NAC carried out with DCF consisted of docetaxel 70 mg/m^2 administered intravenously for 1 h on day 1; 5-fluorouracil 750 mg/m^2 administered as a 24-h continuous intravenous infusion on days 1-5; and cisplatin 70 mg/m^2 administered intravenously for 2 h on day 1. This regimen was conducted every 3 wk. The tumor was evaluated by FDG-PET/CT and endoscopy 1 to 2 wk after completing NAC. Patients underwent esophagectomy 4 to 6 wk after completing NAC.

The clinical disappearance of the primary tumor was evaluated by endoscopy and FDG-PET/CT. Endoscopic tumor disappearance was evaluated according to the Classification of Esophageal Cancer published by the Japan Esophageal Society[16], which describes disappearance as no visible tumor lesions with no mucosa and with an irregular surface, active esophagitis, ulceration and protruding changes with submucosal tumor and the absence of cancer cells in biopsy specimens. The pathological complete disappearance of the primary tumor was defined as grade 3 (pathological complete response).

FDG-PET/CT imaging and diagnosis

Tumors were clinically staged using systematic FDG-PET/CT imaging before and after NAC. The patients were assessed by FDG-PET/CT 1 to 2 wk after completing NAC. The cut-off for primary tumor or LN metastasis was a maximal standardized uptake value (SUV_{max}) of 2.5 as described previously[17-19]. After the NAC, a $SUV_{max} \leq 2.5$ was defined as PET-CMR, and a $SUV_{max} > 2.5$ was defined as non-PET-CMR in this study. We compared clinicopathological factors and short- and long-term outcomes between patients with PET-CMR and non-PET-CMR before and after NAC.

Procedures of esophagectomy

Subtotal esophagectomy with two- or three-field LN dissection was performed for thoracic esophageal cancer. When esophageal cancer was in the upper and middle third of the thoracic esophagus and LN metastasis was present in the superior mediastinum, cervical LN dissection was added. When esophageal cancer was in the lower thoracic esophagus and LN metastasis in the superior mediastinum was not suspected, cervical LN dissection was not necessary. If LN metastasis in the superior mediastinum was revealed pathologically after the esophagectomy, cervical LN dissection was performed post esophagectomy. A gastric tube was subsequently lifted

via the posterior mediastinal route, and high thoracic esophagogastrostomy was performed with a circular stapler. Cervical esophagectomy or pharyngeal-laryngeal-cervical esophagectomy with cervical LN dissection was performed for cervical esophageal cancer.

Statistical analysis

Categorical variables were analyzed using Chi-square tests, and continuous variables were analyzed using unpaired *t*-tests. Survival outcomes were evaluated for 70 consecutive patients surgically treated before June 2013 and who were followed up for at least 3 yrs. Survival was analyzed using Kaplan-Meier curves and compared using log-rank tests. Relapse-free survival (RFS) was defined as the interval between the date of surgery and the first event (recurrence or death from any cause) or the most recent follow-up evaluation. OS was defined as the interval between the date of surgery and death from any cause or the most recent follow-up evaluation. The effects of various clinicopathologic parameters on survival were assessed using univariate analysis and multivariate Cox proportional hazards analysis. Covariates with a P value < 0.05 in the univariate analysis were entered into the multivariate analyses. All data were statistically analyzed using SPSS software version 24 (IBM Corporation, Armonk, NY, United States).

RESULTS

Patient characteristics

Twenty-two (38%) of the 58 patients were diagnosed as having CMR with FDG-PET/CT (PET-CMR group), and 36 patients (62%) were diagnosed as not having CMR (non-PET-CMR group). Patient characteristics are summarized in Table 1. There were no significant differences in age, sex, primary tumor location and LN and distant metastasis before NAC between the two groups. Downstaging was observed in 15 patients (68.1%) in the PET-CMR group and in 17 patients (56.7%) in the non-PET-CMR group. There were no patients with clinical complete response in either group. The SUV_{max} of the primary tumor before NAC was 16.6 ± 6.5 in the PET-CMR group and 17.5 ± 6.5 in the non-PET-CMR group. FDG accumulation in LNs before NAC was observed in 14 patients (63.6%) in the PET-CMR group and in 21 patients (58.3%) in the non-PET-CMR group. There were no significant differences in FDG accumulation in the primary tumor or in LNs between the two groups.

Surgical outcomes

Surgical outcomes are shown in Table 2. There were no significant differences in operative procedures and fields of LN dissection between the two groups. There were 7 cases (19.4%) of noncurative resection (R1) in the non-PET-CMR group, whereas all esophagectomies were curatively performed in the PET-CMR group (P = 0.027). Blood loss was greater in the non-PET-CMR group compared to the PET-CMR group (836 ± 540 mL *vs* 559 ± 219 mL, P = 0.026). Bronchial injury requiring repair of the bronchus occurred in one patient during surgery in each group. There were no significant differences in postoperative complications (Clavien-Dindo classification ≥ 3) between the two groups.

Pathological findings

Pathological findings are shown in Table 3. Pathological grade 3 was achieved in 9 of 22 patients (41%) in the PET-CMR group and in 3 of 36 patients (8%) in the non-PET-CMR group. The non-PET-CMR group showed more aggressive behavior in terms of pT, pStage, lymphovascular invasion and pathological grade. However, there was no significant difference in pN between the two groups.

Accuracy of PET-CMR

The accuracy of PET-CMR is shown in Table 4. The accuracy of PET-CMR for the T factor was 69.0%. Similarly, sensitivity was 68.2%, and specificity was 71.4%. The accuracy, sensitivity and specificity of PET-CMR for the N factor were 65.5%, 41.9%, and 92.6%, respectively. When the T and N factors were combined, the accuracy was 72.4%, sensitivity was 71.7%, and specificity was 75.0%.

RFS, OS and recurrence

Five-year RFS and 5-yr OS are shown in Figure 1. The mean follow-up period was 44.3

Table 1 Patient characteristics

	PET-CMR, *n* = 22	Non-PET-CMR, *n* = 36	*P* value
Age, mean	65.9 (48.0-76.0)	65.0 (47.0-80.0)	0.82
Male/Female	20/2	33/3	0.41
Comorbidity	15	28	
Location			
Ce	1	4	0.58
Ut	4	3	
Mt	10	17	
Lt	7	12	
Before NAC			
cStage			
cT			
T1	2	0	0.044
T2	6	3	
T3	13	32	
T4	1	1	
cN			
N0	5	9	0.27
N1	12	14	
N2	4	13	
N3	1	0	
cM			
M0	17	28	0.96
M1	5	8	
cStage			
I	3	4	0.94
II	4	5	
III	10	19	
IVA	5	8	
IVB	0	0	
Primary, SUV_{max} mean	16.4 ± 6.5	15.7 ± 6.5	0.98
LN+	14 (63.6%)	21 (58.3%)	0.69
After NAC			
CT-Stage			
T			
T1	9	2	0.0058
T2	12	26	
T3	1	6	
T4	0	2	
N			
N0	21	21	0.0081
N1	1	8	

N2	0	7	
N3	0	0	
M			
M0	22	36	-
M1	0	0	
Stage			
0	5	0	0.0017
I	4	1	
II	12	23	
III	1	10	
IVA	0	2	
IVB	0	0	
Down stage	15	17	0.119

Ce: Cervical esophagus; CT: Computed tomography; LN: Lymph node; Lt: Lower thoracic esophagus; Mt: Middle thoracic esophagus; NAC: Neoadjuvant chemotherapy; PET-CMR: Positron emission tomography-complete metabolic response; SUV_{max}: Maximal standardized uptake value; Ut: Upper thoracic esophagus

mo. Five-year RFS of the PET-CMR group was significantly more favorable than that of the non-PET-CMR group (38.6 ± 17.7 mo *vs* 20.8 ± 17.8 mo, $P = 0.021$). Similarly, 5-yr OS of the PET-CMR group was also significantly more favorable than that of the non-PET-CMR (42.8 ± 14.8 mo *vs* 25.1 ± 16.0 mo, $P = 0.011$). Five patients (22.7%) had postoperative recurrence in the PET-CMR group *vs* 17 patients (47.2%) in the non-PET-CMR group (Table 5). There were no significant differences in the distribution of the sites of recurrence between the two groups ($P = 0.66$).

Univariate and multivariate analyses of prognostic factors

Various clinicopathologic factors and PET findings of the primary tumor and LNs were evaluated as prognostic factors using Cox regression models (Table 6 and 7). The univariate analysis of RFS showed that tumor difference, pathologic T, N, stage, complete response, CT-PET negative T and N and PET-CMR were statistically significant. The multivariate analysis subsequently selected CT-PET negative N (hazard ratio: 22.570; 95% confidence interval: 4.69-177.80; $P < 0.01$) as the independent covariate for RFS. CT-PET-T and CT-PET-CMR were excluded from the multivariate analysis due to multicollinearity with CT-PET-N. The univariate analysis of OS showed pathologic T, N, M, stage, complete response and CT-PET negative T and N to be statistically significant. Then, the multivariate analysis subsequently selected CT-PET negative N (hazard ratio: 8.268; 95% confidence interval: 1.74-63.60; $P < 0.01$) as the only independent covariate for OS.

DISCUSSION

NAC is a standard strategy for locally advanced esophageal cancer, and triplet NAC with DCF instead of CF has been applied in clinical practice due to its effectiveness. FDG/PET-CT is useful for assessing tumor progression before surgery, but the usefulness of FDG/PET-CT as a prognostic predictor has not been clarified. In this study, we confirmed that the patients with PET-CMR had a more favorable prognosis than those with non-PET-CMR after NAC with DCF. Furthermore, the presence of PET-positive LNs after NAC could be an independent prognostic factor.

CT is a widely used diagnostic modality to determine staging in esophageal cancer. The sensitivity and specificity of CT for the detection of LN metastasis in esophageal cancer were reported to be 38.57% and 93.93%, respectively[20]. In contrast, the accuracy of CT in detecting regional LN metastasis in esophageal cancer was reported to be unsatisfactory[11]. Similarly, the sensitivity, specificity and accuracy of CT for the detection of LN metastasis in esophageal cancer in our study were 34.4%, 84.6%, 56.9%, respectively. ^{18}F-FDG-PET/CT has moderate/low sensitivity and high/mo-

Table 2 Surgical outcomes

	PET-CMR, *n* = 22	Non-PET-CMR, *n* = 36	*P* value
Esophagectomy procedures			
Subtotal	20	32	0.31
Cervical	1	0	
Pharyngeal laryngeal	1	4	
LN dissection			
Cervical	1	2	0.89
2 fields	5	10	
3 fields	16	24	
Harvested LN[1]	72 ± 7	73 ± 5	0.91
Blood loss in mL[1]	559 ± 219	836 ± 540	0.026
OP time in min[1]	527 ± 105	580 ± 121	0.092
Curability			
R0	22	29	0.027
R1	0	7	
Intraoperative complication			
Bronchus injury	1	1	0.72
Postoperative complication			
All	14	22	0.85
Anastomotic leakage[2]	1	1	0.72
Anastomotic stenosis[2]	2	3	0.92
Recurrent nerve palsy[2]	7	12	0.91
Respiratory[2]	3	8	0.42
Others[2]	2	3	0.92
Surgical mortality	0	0	-

[1]mean ± SD.
[2]Clavien-Dindo Classification ≥ 3.
LN: Lymph node; OP: Operation; PET-CMR: Positron emission tomography-complete metabolic response.

derate specificity for the detection of regional nodal metastasis in patients with esophageal squamous cell carcinoma. Furthermore, previous reports have shown that FDG-PET/CT is useful for diagnosing metastatic LN and distant metastasis and for detecting recurrent esophageal cancer after surgery. An FDG-PET/CT SUV_{max} > 2.6 and a CT attenuation pattern were reported to accurately detect regional LN metastasis with an accuracy of 84.5%, sensitivity of 70.7% and specificity of 86.7%[21,22]. Novel PET/CT using 4′-(methyl-11C) thiothymidine was reported, and the accuracy, sensitivity and specificity of 4′-(methyl-11C) thiothymidine-PET/CT were 92.3%, 90.0% and 93.8%, respectively[23], whereas those of PET-CMR in our study were 72.4%, 71.7% and 75.0%, respectively. These results are equivalent to previous reports, but assessment of the N factor with PET-CMR can result in slightly different interpretations. The sensitivity and specificity of PET-CMR for the N factor are 41.9% and 92.6%, respectively, indicating that even if FDG accumulation in LNs is positive, the result may not necessarily be accurate. However, if FDG accumulation in LNs is negative, negative involvement of cancer can be determined with high probability. Therefore, PET/CT could be a useful tool for the detection of LN metastasis in esophageal cancer compared with CT.

In the present study, PET-CMR was an independent prognostic predictor in the univariate analysis of RFS. One of the reasons is that curative resection was performed in all cases of PET-CMR group, whereas noncurative resection was performed in only

Table 3 Pathological findings

	PET-CMR, *n* = 22	Non-PET-CMR, *n* = 36	*P* value
T			
T0	9	5	< 0.0001
T1	11	4	
T2	1	9	
T3	1	12	
T4	0	6	
N			
N0	13	14	0.052
N1	7	7	
N2	2	8	
N3	0	7	
M			
M0	20	32	0.81
M1	2	4	
Stage			
0	9	3	0.033
I	3	8	
II	8	8	
III	0	10	
IVA	2	4	
IVB	0	3	
Ly+	4	22	0.001
v+	1	15	0.002
Pathological grade			
0	0 (0%)	3 (8%)	0.029
1a	4 (18%)	14 (39%)	
1b	3 (14%)	6 (17%)	
2	6 (27%)	10 (28%)	
3	9 (41%)	3 (8%)	

Ly: Lymphatic invasion; PET-CMR: Positron emission tomography-complete metabolic response; v: Venous invasion.

19% of cases in the non-PET-CMR group. Intraoperative blood loss may also have affected RFS. However, PET-CMR was not an independent prognostic predictor in the multivariate analysis of OS. We speculate that this indicates that OS might depend on the therapeutic regimen used to treat recurrence.

PET-negative LNs were confirmed as one prognostic predictor. In general, LN metastasis occurring after NAC is the most important prognostic factor in patients with resectable esophageal cancer[24]. However, the value of FDG-PET/CT in predicting pathologic LN metastasis and survival among patients with esophageal cancer is controversial. FDG uptake in primary tumors has been helpful in evaluating responses to neoadjuvant therapy and survival[25,26]. It was reported that the metabolic nodal response of esophageal cancer after NAC could be an independent predictor[27]. That report analyzed pathological nodal status and metabolic nodal status as a single factor, so metabolic nodal status alone may not be an independent prognostic factor. In the present study, PET-negative LNs after NAC were an independent prognostic factor in the univariate and multivariate analyses. We previously reported that some

Table 4 Accuracy of positron emission tomography complete metabolic response

	TP	FP	TN	FN	Accuracy	Sensitivity	Specificity
T factor	30	4	10	14	69.0%	68.2%	71.4%
N factor	13	2	25	18	65.5%	41.9%	92.6%
T and N	33	3	13	9	72.4%	71.7%	75.0%

FN: False negative; FP: False positive; TN: True negative; TP: True positive.

Table 5 Sites of recurrence

	PET-CMR, *n* = 5	Non-PET-CMR, *n* = 17	*P* value
Local	0	4	0.66
Distant	5	16	
Extraregional LN	3	6	
Liver	0	5	
Bone	1	4	
Lung	1	3	
Pleura	0	2	

LN: Lymph node; PET-CMR: Positron emission tomography-complete metabolic response.

molecular biomarkers might be predictive factors for esophageal cancer in patients treated with NAC[28]. If these predictive molecular biomarkers can be clarified further, it is possible that patients with PET-negative LNs after NAC could avoid esophagectomy and undergo strict surveillance.

There are some limitations in this study. First, this is a retrospective study with a small sample size. Because NAC with DCF is a relatively novel regimen and was only introduced in our department in 2013, the follow-up periods are currently limited. We are presently collecting prospective data. Then, larger sample size studies are needed to confirm our results in the future. Second, although neoadjuvant CRT is considered a standard option for esophageal cancer in western countries, a randomized control trial (JCOG1109) is now underway in Japan; therefore, we do not yet have enough data on neoadjuvant CRT. Third, the patients who received definitive CRT after NAC were excluded from this study. Further investigations that include patients treated with neoadjuvant CRT and definitive CRT are necessary.

CONCLUSION

PET-CMR after NAC is a favorable prognostic factor for esophageal cancer in terms of RFS. Evaluation by PET-CT after NAC could be useful in clinical decision making for esophageal cancer. Furthermore, in limited patients with PET-negative LNs after NAC, a watch-and-see strategy may be one possible option to avoid unnecessary esophagectomy in the future.

Table 6 Univariable and multivariable analyses of prognostic factors in esophageal cancer

	Relapse-free survival					
	Univariate			Multivariate		
	RR	95%CI	*P* value	RR	95%CI	*P* value
Age	0.982	0.935-1.033	0.468			
Male/Female	2.211	0.638-5.920	0.1885			
Location, MtLtAe/CeUt	1.230	0.406-3.090	0.689			
Tumor grade, other/por	2.612	1.050-6.026	0.040	1.412	0.498-3.785	0.503
pT, 0▪1▪2/3▪4	4.554	1.983-10.770	0.0004	1.195	0.433-3.432	0.734
pN, 0/1▪2▪3	5.287	2.062-16.320	0.0003	3.172	1.074-10.710	0.036
pM, 0/1	2.543	0.726-6.977	0.131			
pStage, 0▪1▪2/3▪4	8.655	3.652-22.060	< 0.0001	2.449	0.356-16.800	0.357
pCR (primary tumor), CR (+)/nonCR (-)	5.199	1.501-32.770	0.0063	1.878	0.337-15.060	0.487
CT-PET-T, (-)/(+)	2.561	1.059-7.120	0.0036			
CT-PET-N, (-)/(+)	10.810	4.251-28.160	< 0.0001	22.570	4.694-177.800	< 0.0001
CT-PET-CMR, (-)/(+)	2.523	1.034-7.063	0.041			

Ae: Abdominal esophagus; Ce: Cervical esophagus; CI: Confidence interval; CR: Complete response; CT-PET-N: Computed tomography-positron emission tomography negative N; CT-PET-T: Computed tomography-positron emission tomography negative T; Lt: Lower thoracic esophagus; Mt: Middle thoracic esophagus; pCR: Pathological complete response; PET-CMR: Positron emission tomography-complete metabolic response; por: Poorly differentiated carcinoma; RR: Relative risk; Ut: Upper thoracic esophagus.

Table 7 Univariable and multivariable analyses of prognostic factors in esophageal cancer

	Overall survival					
	Univariate			Multivariate		
	RR	95%CI	*P* value	RR	95%CI	*P* value
Age	1.036	0.972-1.112	0.285			
Male/Female	1.302	0.204-4.683	0.736			
Location, MtLtAe/CeUt	1.057	0.242-3.291	0.931			
Tumor grade, other/por	1.603	0.499-4.466	0.403			
0▪1▪2/3▪4	8.167	2.912-25.340	< 0.0001	1.644	0.430-8.039	0.486
pN, 0/1▪2▪3	5.653	1.803-24.800	0.0021	0.924	0.045-7.556	0.946
pM, 0/1	5.043	1.354-15.690	0.019	1.518	0.302-6.353	0.588
pStage, 0▪1▪2/3▪4	15.52	5.062-58.400	< 0.0001	5.760	0.415-160.800	0.197
pCR (primary tumor), CR (+)/nonCR (-)	3.202	0.887-20.470	0.0793			
CT-PET-T, (-)/(+)	3.227	1.106-11.680	0.0313	1.450	0.307-6.866	0.630
CT-PET-N, (-)/(+)	25.270	6.361-168.100	< 0.0001	8.268	1.748-63.630	0.006
CT-PET-CMR, (-)/(+)	2.927	0.996-10.660	0.0510			

Ae: Abdominal esophagus; Ce: Cervical esophagus; CI: Confidence interval; CR: Complete response; CT-PET-N: Computed tomography-positron emission tomography negative N; CT-PET-T: Computed tomography-positron emission tomography negative T; Lt: Lower thoracic esophagus; Mt: Middle thoracic esophagus; pCR: Pathological complete response; PET-CMR: Positron emission tomography-complete metabolic response; por: Poorly differentiated carcinoma; RR: Relative risk; Ut: Upper thoracic esophagus.

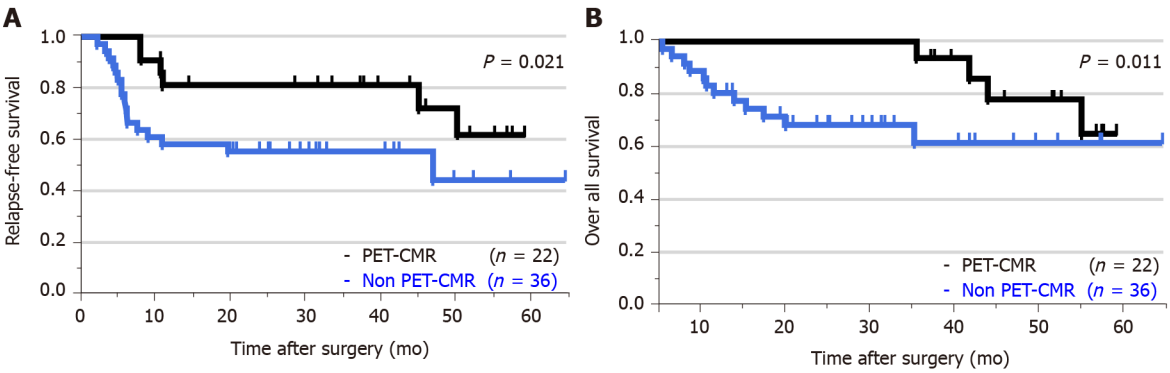


Figure 1 Survival curves after esophagectomy with neoadjuvant chemotherapy. A: Relapse-free survival rates of the positron emission tomography-complete metabolic response (PET-CMR) group and non-PET-CMR group (P = 0.021); B: Overall survival rates of the PET-CMR group and non-PET-CMR group (P = 0.011). PET-CMR: Positron emission tomography-complete metabolic response.

ARTICLE HIGHLIGHTS

Research background

Recent studies have shown that ^{18}F-fluorodeoxyglucose-positron emission tomography (FDG-PET) can determine the degree of metabolic activity in tumor cells and can improve tumor staging for patients with esophageal cancer. Furthermore, several studies have shown that FDG-PET/computed tomography (CT) (FDG-PET/CT) is useful for diagnosing metastatic lymph nodes and distant metastasis and for detecting the recurrence of esophageal cancer after surgery. However, the value of FDG-PET/CT to predict survival in patients with esophageal cancer is controversial.

Research motivation

Accurate assessment of cancer remnants after neoadjuvant chemotherapy (NAC) in esophageal cancer may lead to a watch-and-see treatment strategy to avoid surgery.

Research objectives

We aimed to evaluate the value of PET complete metabolic response in esophageal cancer following NAC as a prognostic predictor.

Research methods

We reviewed data from 70 consecutive patients with squamous cell esophageal cancer who were preoperatively evaluated with FDG-PET/CT before and after NAC with docetaxel, cisplatin and 5-fluorouracil between June 2013 and December 2017 at Oita University. Those who received definitive radiotherapy/chemotherapy after NAC (n = 11) and underwent esophagectomy in another hospital (n = 1) were excluded. Thus, 58 patients received esophagectomy after NAC and were enrolled in this retrospective cohort study.

Research results

Five-year relapse-free survival and overall survival (OS) in the PET complete metabolic response group were significantly more favorable than those in the non-PET complete metabolic response group (38.6 mo *vs* 20.8 mo, P = 0.021, 42.8 mo *vs* 25.1 mo, P = 0.011, respectively). The univariate analysis of OS showed pathologic T, N, M, stage, complete response and CT-PET negative T and N to be statistically significant. Then, the multivariate analysis subsequently selected CT-PET negative N (hazard ratio: 8.268; 95% confidence interval 1.74-63.60; P < 0.01) as the only independent covariate for OS.

Research conclusions

The multivariate analysis subsequently selected CT-PET negative N (hazard ratio: 8.268; 95% confidence interval: 1.74-63.60; P < 0.01) as the only independent covariate for OS.

Research perspectives

In limited patients with PET-negative lymph nodes after NAC, a watch-and-see

strategy may be one possible option to avoid unnecessary esophagectomy in the future.

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CASE REPORT

Hepatocellular carcinoma with biliary and neuroendocrine differentiation: A case report

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Author contributions: Dimopoulos YP, Ozdemirli M, He AR, and Winslow ER reviewed the literature, wrote the manuscript, and revised the manuscript; Dimopoulos YP and Ozdemirli M were involved in the pathology side of managing the patient; Winslow ER performed the patient's surgery and was involved in the patient's pre- and post-surgical care and follow-up; He AR was the oncologist and was involved in the patient's care; all authors issued final approval for the version to be submitted.

Informed consent statement: At the time of preparation of this case report, the patient had passed away. Thus, informed consent was not able to be obtained. However, utmost care was shown during the preparation of the case report to not reveal any personally identifiable protected health information. Also, per the MedStar Office of Research integrity, case reports including 3 or fewer individuals do not meet the U.S. Department of Health and Human Services (DHHS) definition of

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Abstract

BACKGROUND

Liver tumors with dual differentiations [combined hepatocellular carcinoma (HCC) and cholangiocarcinoma] are common. However, liver tumors that exhibit hepatocellular, biliary, and neuroendocrine differentiation are exceedingly rare, with only three previous case reports in the literature.

CASE SUMMARY

A 65-year-old female with a previous history of hepatitis C and a distant history of low grade, well-differentiated rectal neuroendocrine tumor was found to have two liver lesions in segment 4 and segment 7 on imaging. Serum alpha-fetoprotein and chromogranin A were elevated. Biopsy of the larger lesion in segment 4 revealed a high-grade tumor, with morphologic and immunohistochemical features of a neuroendocrine tumor. Given the previous history of rectal neuroendocrine tumor, imaging investigation, serologic markers, and biopsy findings, metastatic neuroendocrine tumor was considered. Subsequent regional resection of these hepatic lesions revealed the segment 4 lesion to be a HCC with additional biliary and neuroendocrine differentiation and the segment 7 lesion to be a cholangiocarcinoma with neuroendocrine differentiation. Follow-up of the patient revealed disease recurrence in the dome of the liver and metastasis in retro-pancreatic lymph nodes. The patient eventually expired due to complications of chemotherapy.

CONCLUSION

HCC cases with additional biliary and neuroendocrine differentiation are exceedingly rare, posing a diagnostic challenge for clinicians and pathologists.

research and therefore do not require Institutional Review Board (IRB) review but are still subject to Health Insurance Portability and Accountability Act of 1996 (HIPAA) requirements. No Protected Health Information (PHI) was disclosed during the preparation of the case report.

Conflict-of-interest statement: All authors declare that they have no conflicts of interest to disclose.

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Core Tip: Hepatocellular carcinoma (HCC) exhibiting additional biliary and neuroendocrine differentiation are exceedingly rare, with only three previous case reports in the literature. We present a case of HCC with this triple differentiation, which developed in a patient with a history of previously excised rectal neuroendocrine tumor. This case highlights the diagnostic difficulties encountered during the evaluation of these patients, especially in cases where patients have a previous history of neuroendocrine tumors elsewhere in the body. The aggressive nature of these tumors is demonstrated in addition.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent subtype of primary liver cancer (PLC), accounting for 70%-85% of all PLC cases[1]. Traditionally, HCC has been separated from cholangiocarcinoma (CC), with differences in clinical management, treatment, and prognostic stratification of patients[2]. PLCs exhibiting multiple differentiation pathways have been described. For example, PLCs of both hepatocytic and biliary differentiation (combined HCC-CC (cHCC-CC)) are relatively common, with an incidence ranging between 0.4%-14.2%[1,3]. Apart from cHCC-CC, other rare PLCs with combined differentiations exist. Tumors with an HCC and neuroendocrine carcinoma (NEC) differentiation have been reported previously[4]. Additionally, cases of CC of the intra- and extrahepatic biliary tree with neuroendocrine differentiation have been designated as mixed adeno NEC (mANEC)[5,6].

Hepatocellular tumors exhibiting three differentiation pathways are exceedingly rare. The combination of hepatocytic, biliary and neuroendocrine differentiation has been previously described in 3 patients[7,8] (Table 1). We report, herein, an additional case of similar tumor arising in a 65-year-old female.

CASE PRESENTATION

Chief complaints

A 65-year-old female presented with vague symptoms of peri-umbilical and epigastric abdominal pain, poor appetite, unintentional weight loss of 10 pounds, and night sweats.

History of present illness

Computed topography (CT) scan performed during the work-up of the patient revealed two liver lesions.

History of past illness

The patient had a history of neuroendocrine tumors in the rectum. Specifically, in a screening colonoscopy performed in 2012, a 1 cm polypoid lesion was seen in the rectum. This was removed endoscopically, with saline injection and hot snare cautery. Review of the histological slides revealed a low-grade, well-differentiated neuroendocrine tumor ("carcinoid") with no evidence of lymphatic or vascular invasion. Resection margins were not able to be assessed on this excision pathologically. On subsequent colonoscopy in 2017, a more extensive endoscopic

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mucosal resection was performed in the region of the previously identified carcinoid, with final margins negative on pathologic examination. No recurrence in the region of the rectum or additional neuroendocrine tumors in her colon were seen on subsequent colonoscopies, and no liver lesions were seen on CT imaging.

The patient additionally had a previous medical history of hypothyroidism (treated with levothyroxine) and hypertension (treated with clonidine, amlodipine, and hydrochlorothiazide-triamterene).

Physical examination

Mild diffuse tenderness was found on abdominal examination. No masses or hepatomegaly were detected. The rest of the examination was unremarkable.

Laboratory examinations

Serum chromogranin and alpha-fetoprotein (AFP) were both elevated, at 450 ng/mL (normal range: < 93 ng/mL) and 2198 ng/mL (normal range: 10-20 ng/mL), respectively. The percentage of AFP binding to Lens culinaris agglutinin (AFP-L3%) was also elevated (86.6%). The patient was found to be positive for hepatitis C virus (HCV) by polymerase chain reaction. HCV genotype was determined to be 1a. Laboratory examinations at the time were additionally notable for mildly increased aspartate aminotransferase (72 U/L; normal range: 0-35 U/L) and alanine transaminase (86 U/L; normal range: 19-25 U/L).

Imaging examinations

Standard dynamic CT imaging of the abdomen and pelvis with intravenous contrast revealed a total of two hepatic masses: A 2.2 cm × 2.1 cm peripherally enhancing mass in the left lobe (segment 4) with central hypodensity, and a 1.4 cm × 1 cm lesion in the right lobe (segment 7) that was low density (Figure 1). Additionally, prominent portacaval lymph nodes were noted.

To characterize the lesions further, magnetic resonance imaging (MRI) with gadolinium-based contrast was subsequently performed, with multiple pulse sequences, including T1 and T2 weighted and in phase/opposed phase gradient-echo images. Diffusion imaging was also performed. These images showed a 2.7 cm × 2.5 cm × 2.3 cm T2 hyperintense lesion in the left lobe (segment 4) with peripheral rim-like enhancement on the arterial phase and persistent enhancement on the venous phase (Figure 1). The lesion in the right lobe (segment 7) was 1.5 cm × 1.5 cm × 1.1 cm and similarly T2 hyperintense but was too small to evaluate its enhancement characteristics adequately.

Fluorodeoxyglucose (FDG) positron emission tomography (PET) showed FDG avidity in the left lobe lesion (standard uptake value of 6.6) but no avidity in the right lobe lesion. No extrahepatic FDG avidity in the nodal basins or pelvis was seen.

Gallium-68 dotatate PET scan was also performed and showed no tracer uptake in the rectum, liver, or portal lymph nodes.

Further diagnostic work-up

The left lobe segment 4 lesion of the liver was biopsied under CT guidance, with final pathology findings being consistent with a high-grade neuroendocrine tumor (due to morphology and synaptophysin expression detected by immunohistochemistry). A Ki-67 immunohistochemical investigation revealed 30% of the cells to be positive. Concurrent biopsy of uninvolved hepatic parenchyma revealed absence of cirrhosis or significant fibrosis. A repeat MRI scan performed showed an interval increase in the size of the segment 4 lesion, which measured 4.1 cm × 3.6 cm × 2.3 cm. Chest CT showed no evidence of metastatic disease.

A combination of clinical, imaging, and pathologic findings raised the possibility of metastatic neuroendocrine tumor to the liver at the time, with the main differential diagnosis considered being a primary hepatic tumor (most likely HCC). Given that there was no indication of extra-abdominal spread on imaging studies and the absence of cirrhosis in the hepatic parenchyma, the decision to proceed with segmental resection of the two hepatic lesions with portacaval lymph node sampling was made, as this was felt to be beneficial for either differential diagnoses considered. Furthermore, plans were made to treat the patient's HCV infection following the surgical intervention.

Surgical intervention and resection

A staging laparoscopy was performed and revealed no evidence of metastatic disease outside of the liver and confirmed the absence of cirrhosis. Open exploration with

Table 1 Hepatic tumor cases reported to have triple (hepatocellular, cholangiocytic, and neuroendocrine) differentiation

Cases of liver tumor with triple differentiation described in the literature	Ref.
Chinese male patients (n = 2; average age: 57.5 yr). Both were positive for hepatitis B and exhibited hepatic tumors with triple differentiation.	He *et al*[8], 2013
An otherwise healthy 19-year-old Caucasian male developed a large hepatic tumor showing triple differentiation.	Beard *et al*[7], 2017
A 65-year-old female with a previous history of hepatitis C and rectal "carcinoid" developed a hepatic tumor with triple differentiation.	Current case report

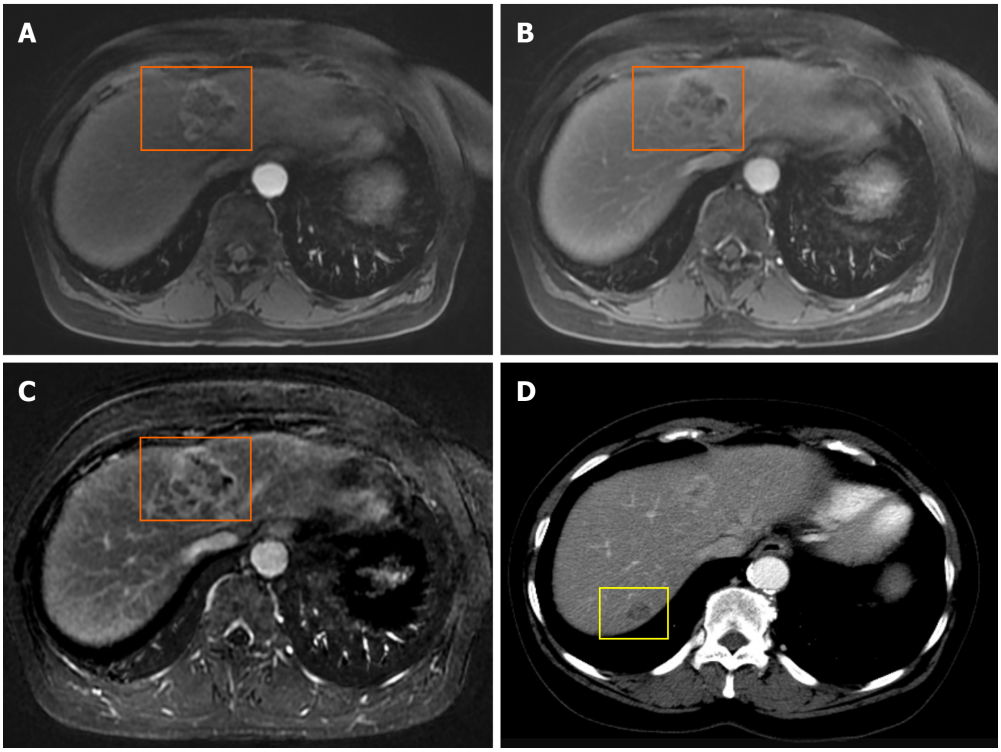


Figure 1 Magnetic resonance imaging and computed tomography imaging. A-C: Magnetic resonance imaging with gadolinium was performed and demonstrated the lesion in segment 4 (orange box); D: Computed tomography imaging showed the lesion in segment 7 most clearly (yellow box).

intraoperative ultrasound revealed only the two known liver lesions. Anatomic resection of segment 4 and nonanatomic resection of the lesion in segment 7 were performed, as well as a portal lymphadenectomy.

Pathologic examination of the left lobe segment 4 resection specimen revealed a segment of red-brown liver parenchyma measuring 10.2 cm × 5.1 cm × 4.1 cm. On serial sectioning, a tan-white to tan-green mass measuring 4.7 cm × 4.0 cm × 2.3 cm was identified. The surgical resection margin was negative. Microscopic evaluation of this tumor revealed a poorly differentiated malignant tumor with variegated and intermixed histology. Based on morphology, foci resembling HCC, CC, and neuroendocrine tumor were identified (Figure 2). Immunohistochemical analysis showed the malignant cells to be focally positive for AFP, cytokeratin (CK)-19, CK-7, CK-18, CK-20, p53, polyclonal carcinoembryonic antigen, Hep-Par-1, arginase 1, CDX-2, as well as focally positive for synaptophysin and neuron-specific enolase (Figure 3). Ki-67 proliferation index was high, estimated at approximately 60%. The tumor cells were negative for chromogranin, prostatic acid phosphatase, and cancer antigen 19-9 (CA19-9).

Pathologic examination of the right lobe segment 7 resection specimen revealed a 1.5 cm × 1.5 cm × 1.0 cm segment of hepatic parenchyma, which was serially sectioned to reveal a 1.0 cm × 0.5 cm × 0.5 cm white nodule. The surgical resection margin was negative. Histologic examination of the nodule revealed malignant cells forming glands on a sclerotic background (Figure 4). Immunohistochemical analysis (Figure 4) revealed the malignant cells to be positive for CK-18, CK-19, CK-7, carcinoembryonic antigen, synaptophysin (strong diffuse), chromogranin (scattered and weak), and CDX-2 (scattered and weak). Ki-67 proliferation index was approximately 20%. The

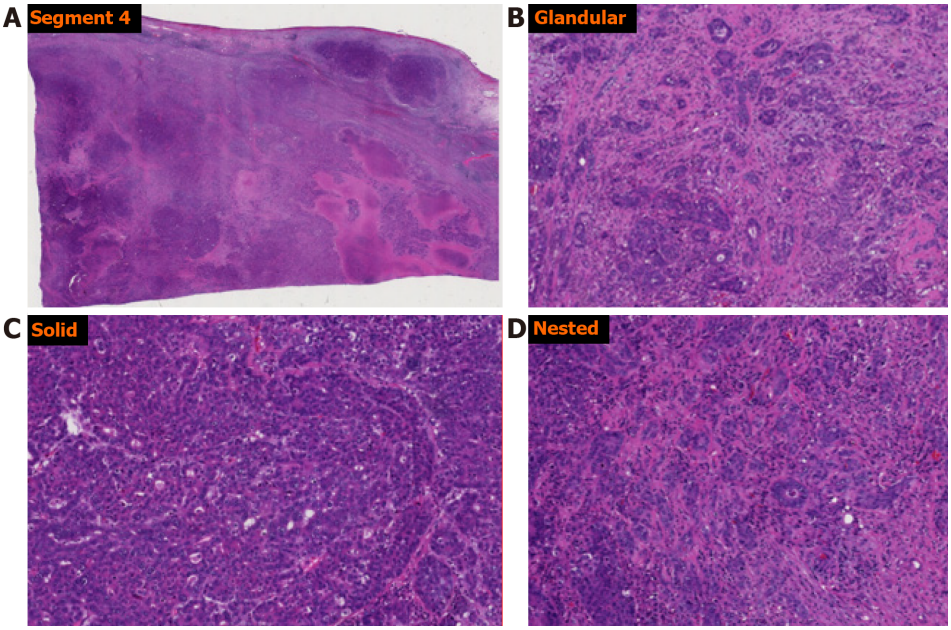


Figure 2 Histologic examination. A: The larger, segment 4 liver lesion revealed a tumor with variegated histology and regions of necrosis; B-D: On closer examination, regions with glandular (B), solid (C), and nested (D) patterns were identified on morphology.

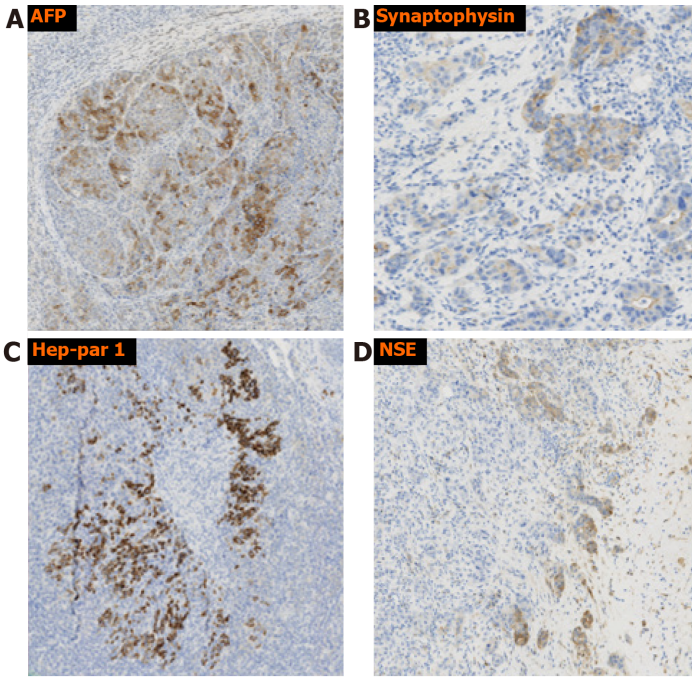


Figure 3 Immunohistochemical investigation of the larger, segment 4 liver lesion. The tumor showed variable and focal positivity for alpha-fetoprotein, Hep-par-1, as well as synaptophysin and neuron-specific enolase. A: Alpha-fetoprotein; B: Synaptophysin; C: Hep-par 1; D: Neuron-specific enolase. AFP: Alpha-fetoprotein; NSE: Neuron-specific enolase.

cells were negative for AFP, CD56, Hep-Par-1, and arginase 1.

Intra-operative consultation on a portacaval lymph node was initially reported as negative. Final pathologic examination of the resected portal lymph nodes revealed a microscopic focus of metastatic HCC in one of the lymph nodes.

MULTIDISCIPLINARY EXPERT CONSULTATION

An oncology consultation was pursued and adjuvant chemotherapy was advocated, following additional imaging investigation.

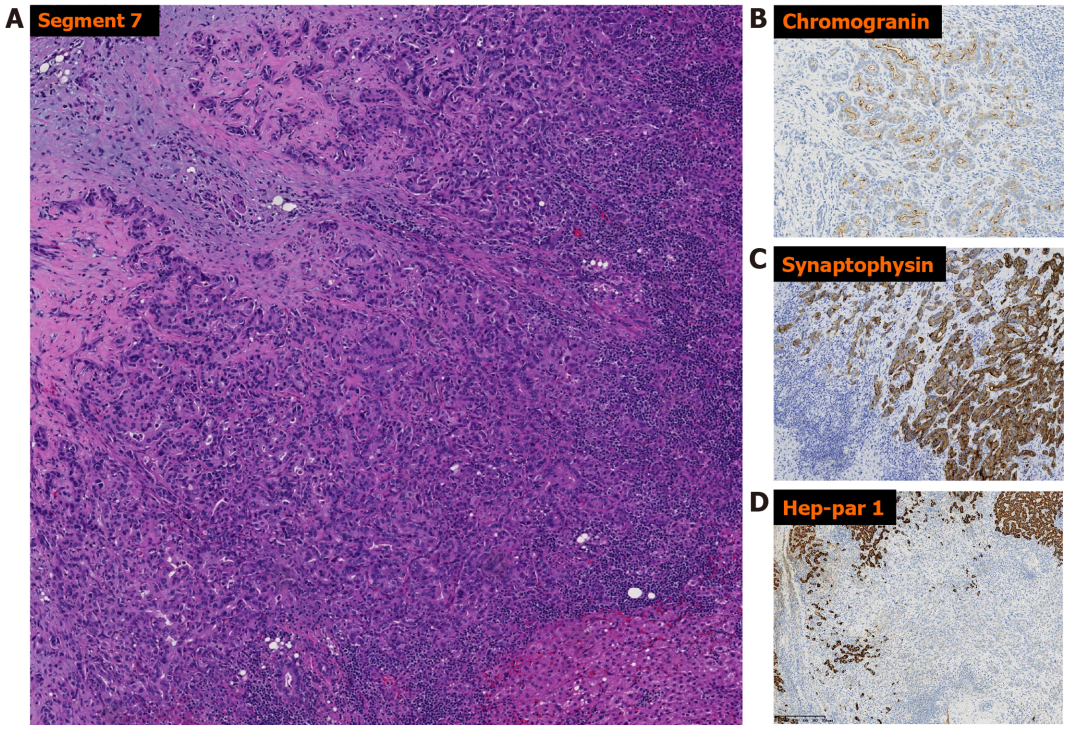


Figure 4 Morphologic evaluation of the smaller, segment 7 liver lesion. A: A predominantly glandular morphology was revealed; B-D: On immunohistochemical investigation, the cells were found to be positive for chromogranin and synaptophysin and negative for Hep-par-1.

FINAL DIAGNOSIS

Based on the morphologic and immunophenotypic characteristics, the final diagnosis of HCC with additional biliary and neuroendocrine differentiation was rendered for the left lobe segment 4 mass, with the hepatocellular component being the predominant morphology/immunophenotype observed. The right lobe segment 7 mass was diagnosed as a mANEC. Given the propensity of HCC to metastasize intra-hepatically, the relative size of the two masses (with the left lobe segment 4 mass being larger than the right lobe segment 7 mass), and findings of metastatic HCC to the portacaval lymph node, and the fact that both tumors showed neuroendocrine differentiation, the right lobe segment 7 mass was favored to represent an intra-hepatic metastatic focus of the larger segment 4 mass. The pathologic staging of the patient's tumors was pT2N1.

TREATMENT

Following the resection of the two hepatic tumors, the patient had an uneventful post-surgical recovery and was subsequently discharged home with instructions for post-surgical follow-up.

OUTCOME AND FOLLOW-UP

Immediate postoperative imaging was performed, including an FDG PET/CT, which demonstrated no FDG avid lesions. The patient was followed up by a community oncologist near her home and did not initially receive adjuvant therapy. CT performed 1-mo post-surgery revealed disease recurrence in the hepatic dome. Additionally, an MRI confirmed recurrent disease in the liver dome and new pathologic lymphadenopathy posterior to the pancreatic head. Endoscopic, ultrasound-guided, transgastric fine needle aspiration of these enlarged lymph nodes revealed metastatic adenocarcinoma, consistent with metastasis from the CC component of the patient's tumors.

Formalin fixed, paraffin embedded tissue from the patient's larger tumor (left lobe, segment 4) was submitted for molecular profiling with next-generation sequencing. This revealed that there was no fibroblast growth factor receptor 2 fusion, *IDH1/2* mutation, *BRAF* mutation, or *HER2* amplification. The tumor was found to be microsatellite stable. There was a *CDKN2A* exon 2 p.H83Y mutation and a *TP53* exon 7 p.Y234C mutation. Both pathologic mutations detected would not have any impact on the selection of first-line systemic therapy. Thus, standard first-line palliative systemic therapy with cisplatin and gemcitabine was initiated.

Five months later, while receiving systemic therapy, the patient developed gastrointestinal bleeding (the patient was on anticoagulation for a port-related thrombosis she had developed) and subsequently multiorgan system failure. Imaging obtained on presentation revealed stable disease regarding her relapsed tumor (unchanged size of the retro-pancreatic pathologic lymph node and right hemi-liver recurrence). Unfortunately, the patient passed away secondary to multiorgan failure (see Table 2 for complete timeline of events).

DISCUSSION

Combined PLCs are increasingly being described and prove a diagnostic challenge for clinicians, radiologists, and pathologists. On CT and MRI imaging, our patient's liver lesions exhibited features more consistent with a metastatic lesion rather than a primary HCC (peripheral rim-like enhancement on the arterial phase and persistent enhancement on the venous phase in the patient's tumor *vs* homogenous arterial phase enhancement and washout on the venous phase typically seen in HCC)[9]. It is known however that, based on the predominant histologic component, they may exhibit varying imaging features on CT and MRI[10].

Gallium-68 dotatate PET scan is a widely accepted method for the detection of neuroendocrine tumors and their metastases, with higher reported sensitivity and specificity compared to octreotide scans[11]. Given our patient's history of a rectal carcinoid, this scan was performed in the context of detecting metastatic disease to the liver, which was suspected clinically. In our patient, the scan failed to reveal uptake in the liver lesions; however, due to the possibility of a false negative result, a metastatic neuroendocrine tumor was not excluded[11].

Serum markers are frequently used in the evaluation of liver tumors, with HCC and CC typically showing an increase in serum AFP and CA19-9, respectively. False positive elevations are well represented in the literature[3,12,13]. Increase in the AFP-L3 percentage has been shown to be relatively specific for HCC[14]. Elevations in AFP have also been reported in the context of metastatic neuroendocrine tumors to the liver[15]. Serum chromogranin has been used as a marker for tumors with neuroendocrine differentiation and for the monitoring of enterochromaffin-like hyperplasia secondary to treatment with acid secretion inhibitors or atrophic gastritis[16]. Our patient exhibited elevations in both serum AFP, AFP-L3 percentage, and chromogranin. Serum CA19-9 was not established pre-operatively, but it was shown to not be elevated post-operatively.

An increased serum AFP and HCV positivity raised the differential diagnosis of a primary HCC. The imaging characteristics and the biopsy results of the lesion in the left liver were not consistent with HCC. Initial CT-guided core biopsy findings of a high-grade tumor with neuroendocrine differentiation highlight the sampling error that has been reported in similar cases of combined PLC and the need to sample multiple areas of the tumor if a combined tumor is suspected[3,10,17]. Our patient had a history of low-grade neuroendocrine neoplasm (carcinoid tumor) that was very different from the high-grade tumors of the liver. Theoretically, metastasis was a possibility given the known rate of discordance in tumoral grade for neuroendocrine tumors between the primary lesion and the liver metastasis[18]. Although the lesion in the right liver was not biopsied, it had similar imaging features by CT and MRI and was thought to be similar in etiology. CC was not strongly considered pre-operatively, given the biopsy finding, imaging features, and tumor markers. Summarizing all the information known at the time, the decision was made for resection of both the liver lesions, which we felt was indicated in either of the main differential diagnoses considered. If truly a metastatic neuroendocrine tumor, these were isolated lesions and amenable to complete resection, which is an established guideline-based recommendation. If the lesions were truly primary lesions (HCC or mixed tumor), resection would be indicated in that case as well, given no distant disease and a liver without fibrosis or cirrhosis.

Table 2 Timeline of patient's history and medical interventions

Dates	Summary from initial and follow-up visits	Diagnostic testing	Intervention/results
2012 and 2017	Routine colonoscopy	1 cm polypoid lesion in rectum	Removed endoscopically → well-differentiated neuroendocrine tumor
May/June 2019	Presents with abdominal pain	CT, MRI, FDG/PET → two liver lesions	CT-guided biopsy → poorly differentiated carcinoma with neuroendocrine features
September 2019	Presents for surgical intervention	Presents for surgical intervention	Resection of the 2 liver lesions → Final diagnosis of combined liver tumor
October 2019	Post-surgical follow-up	CT and FDG/PET → New liver lesion in dome	Additional imaging was advocated
March 2020	MRI revealed new portocaval lymphadenopathy	Endoscopic ultrasound-guided FNA of lymph nodes	Lymph nodes positive for metastatic adenocarcinoma
May 2020	Final oncology consultation after imaging		Initiation of cisplatin and gemcitabine chemotherapy
October 2020	Presents to hospital with altered mental status	Found to be in shock due to a combination of GI bleed/sepsis	Patient expired, despite medical treatment

CT: Computed tomography; FDG: Fluorodeoxyglucose; FNA: Fine needle aspiration; GI: Gastrointestinal; MRI: Magnetic resonance imaging; PET: Positron emission tomography.

Histologic examination of the larger (segment 4) tumor in our patient revealed a variegated histology, with regions containing hepatocellular, biliary and neuroendocrine differentiation within the same tumor. By morphology, intermixed components of solid/trabecular architecture with polygonal cells (HCC), glandular architecture (CC), and solid/nested architecture with rounded cells and "salt and pepper" nuclei (neuroendocrine) were seen. Additionally, the triple differentiation was demonstrated immunohistochemically, namely the expression of hepatocellular (HepPar-1, AFP), biliary (CK-7, CK-19) and neuroendocrine (synaptophysin, neuron-specific enolase) markers in the different components of the tumor. This is in contrast with so-called collision tumors, or spatially separate tumor nodules within the same liver, and meets the consensus criteria for a mixed tumor[2,10]. The smaller (segment 7) tumor morphologically resembled a mANEC, with an adenocarcinoma-like architecture and with expression of neuroendocrine markers (synaptophysin). As mentioned previously, given the propensity of HCC for intra-hepatic metastatic spread, the relative size of the two tumors, and the fact that metastasis to the lymph node detected during the surgery had HCC morphology and the fact that both had neuroendocrine differentiation, it was favored that the smaller tumor represented a metastatic focus of the larger tumor. Additionally, morphology and immunophenotype of the tumors excluded metastatic carcinoid tumor, as was suspected initially clinically.

The relative rarity of mixed tumors with three differentiation pathways has made it impossible to reach meaningful conclusions about the risk factors, cell of origin, prognosis, and treatment options. Most reports on these clinical aspects have focused on mHCC-CC or mANEC tumors. It has been postulated that mHCC-CC cases share many of the risk factors with HCC and CC, including cirrhosis and chronic infection with hepatitis B virus or HCV[17]. However, one of the only case reports of a mixed tumor with three differentiation pathways occurred in a young patient with no known identifiable risk factors[7]. Ethnic and environmental influences have been proposed to affect risk[7]. Our patient exhibited chronic infection with HCV without established cirrhosis.

Previous reports have suggested hepatocyte progenitor/stem cells as the likely origin of combined liver tumors[19-21]. As these transformed cells clonally evolve, they may acquire varying and divergent phenotypic and mutational characteristics. This clonal evolution can lead to varying proportions of subtypes in the tumor. The larger tumor in our patient exhibited predominantly hepatocellular differentiation with intermixed regions of biliary and neuroendocrine differentiation, whereas the smaller tumor showed biliary and neuroendocrine differentiation. This is more in keeping with divergent clonal evolution of hepatic stem cells.

As far as prognosis is concerned, mHCC-CCs tend to be considered to have a worse prognosis than HCC but a better prognosis than CC[17]. Previous reports have

highlighted the potential importance of the relative contribution of the different components to the final outcome of the patient[17]. The neuroendocrine component is considered to be more aggressive[4,7]. Evidence for the different biological aggressiveness of the different components exists in the form of microdissection experiments, in which different regions of combined tumors were interrogated for molecular alterations[7]. These showed that the neuroendocrine component of the tumor was associated with detectable mutational change and genomic instability and allelic imbalance involving the 5p and 7p chromosomal regions (regions containing tumor suppressor and proto-oncogenes), corresponding to more aggressive behavior[7]. Additionally, it has been noted that the neuroendocrine component usually is involved in metastatic spread in patients with mixed hepatic tumors[4]. Interestingly, in our patient, the micro-metastasis detected during initial surgery in one of the regional lymph nodes had features of HCC rather than NEC, and the subsequent metastatic focus in another lymph node found post-surgery had features of CC, potentially indicating a heterogeneity and different molecular events present in different patients and clonal subpopulations. This is further supported by findings of microsatellite stability on next-generation sequencing performed on our patient's larger tumor. Granted, microdissection to separate mutational characteristics of the different regions of the tumor was not performed in our patient, and the results most likely are more representative of the predominant component present (*i.e.* HCC).

Treatment is usually attempted with surgical resection, with liver transplant not proving to be superior to a minor or major hepatectomy[3]. The benefits of lymphadenectomy outside of staging are debatable[3]. Other forms of treatment, such as transarterial chemoembolization, radioembolization, or chemotherapy have not been studied adequately and are used in patients who are poor surgical candidates in the context of palliative treatment[3]. The decision on whether to treat a patient with adjuvant therapy or what type of regime for adjuvant therapy is based on the evaluation of the tumor with worst outcome. In our patient, the features of her segment 4 tumor and positive lymph node weighed more on the decision of adjuvant therapy in comparison to the segment 7 tumor. The segment 4 tumor had mixed CC, HCC, and neuroendocrine features with a Ki-67 proliferation index of 60%, which suggests aggressiveness of the tumor and high risk for cancer recurrence. It is hard to determine which tumor component (CC, HCC, or neuroendocrine) will recur. No randomized clinical trial has supported the benefit of adjuvant therapy for HCC so far. Even though one lymph node was found to have microscopic foci of HCC, no adjuvant therapy for HCC was recommended. Adjuvant therapy with cisplatin and gemcitabine for CC and adjuvant therapy with carboplatin/etoposide for neuroendocrine tumor were initially considered. Our patient developed disease recurrence in the form of a hepatic dome lesion and retro-pancreatic lymphadenopathy with biopsy proven CC, detected on imaging following segmental resection of the two tumors, again in keeping with the more aggressive nature of these tumors previously reported. As such, standard first-line palliative systemic therapy with cisplatin and gemcitabine was favored.

CONCLUSION

In conclusion, liver tumors with more than two differentiation pathways are exceedingly rare. A heightened index of suspicion needs to be maintained by clinicians, radiologists, and pathologists to reach the correct diagnosis. Most of the clinical aspects, risk factors, and prognostic data currently are extrapolated from cases of mixed tumors with two pathways of differentiation, mainly mHCC-CC and mANEC tumors. More research is needed to reach adequately solid conclusions concerning appropriate management and prognosis.

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CASE REPORT

Lenvatinib-induced multiorgan adverse events in Hurthle cell thyroid cancer: A case report

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Author contributions: Butt MI is the primary endocrinology physician involved in the care of the patient, and he reviewed the literature and drafted the manuscript; Bakhsh MK is the endocrinology fellow who took informed written consent from the patient and collated and prepared the laboratory data for the manuscript; Nadri QJ offered nephrology consultation and care for the management of renal adverse events; all authors reviewed and approved the final version of the manuscript.

Informed consent statement: The patient provided a written informed consent. We conducted the study in accordance with the guidelines outlined in the declaration of Helsinki. This study was approved by the Institutional Ethics Committee of King Faisal Specialist Hospital and Research Centre (approval No. RAC 2200330).

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Abstract

BACKGROUND

The management of metastatic progressive radioiodine-resistant differentiated thyroid cancer remains challenging for clinicians. The availability of tyrosine kinase inhibitors (TKIs), sorafenib and lenvatinib, within the last decade has expanded treatment options; however, these lead to significant adverse effects, which may curtail their use.

CASE SUMMARY

We report the case of a 47-year-old female with Hurthle cell thyroid cancer who underwent total thyroidectomy followed by radioiodine ablation. During follow-up, she developed noniodine-avid renal and pulmonary metastases. With respect to her pre-existing diabetes, hypertension, and polycystic kidney disease, the tumor board decided against performing renal metastasectomy because of the risk of future renal decline requiring dialysis. Metastases were treated using sorafenib, which provided stability followed by progression within a year. We switched to lenvatinib, which led to disease regression. However, the patient experienced severe adverse effects, including cardiomyopathy, bicytopenia, renal impairment, and the rarely reported nephrotic syndrome. Renal metastasis is a rare manifestation of Hurthle cell thyroid cancer with only two reported cases in literature. We report the experience of our first case of renal metastasis and its treatment with TKIs. This case serves as a reminder of the adverse drug reactions associated with TKI use.

CONCLUSION

We advocate close monitoring of patients' hematological and renal profiles as well as their cardiac status using an echocardiogram.

Key Words: Hurthle cell thyroid cancer; Lenvatinib; Nephrotic syndrome; Vascular endothelial growth factor; Protein kinase inhibitors; Kidney neoplasms/secondary

Checklist (2016), and the manuscript was prepared and revised according to the CARE Checklist (2016).

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Core Tip: The present case study provides a unique learning and case management experience. Our patient had widespread metastasis from a histopathologically low-risk thyroid cancer coupled with two decades of survival despite noniodine-avid metastasis, treatment of renal metastasis with tyrosine kinase inhibitors, and the development of multisystem adverse events, including rare nephrotic syndrome.

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INTRODUCTION

The worldwide annual incidence of thyroid cancer is increasing, and it is the most common cancer that involves the endocrine organs[1]. This increase is thanks to the improved availability and sophistication of imaging technologies, such as high-resolution ultrasonography of the neck. This has led to early detection of small tumors. According to the Surveillance, Epidemiology, and End Results database, the mortality rate of thyroid cancer is exceptionally low at 0.5 per 100000 individuals per year. Moreover, the 5-year survival rate of thyroid cancer is 98.3% because of early detection as well as a predominantly localized disease[2]. In Saudi Arabia, the incidence of thyroid cancer subtypes show a pattern similar to the rest of the world, with papillary thyroid cancer at 85%, follicular thyroid cancer at 5.7%, and medullary thyroid cancer at 3.1%. However, the incidence of thyroid cancer among Saudi citizens in 2015 was 8.5% of all newly diagnosed cancers[3]. This contrasts with a much lower annual rate of 2.9% in the United States[2]. The exact reason for this is unknown. However, possibilities include iodine deficiency in the region, exposure to depleted uranium in the previous Gulf war, and increasing prevalence of obesity in the area.

The prognosis for patients with radioactive iodine (RAI)-avid metastatic disease remains good, with a 56% 10-year survival in avid metastasis *vs* 10% 10-year survival in noniodine-avid metastasis[4]. Over time, nearly two-thirds of these distant metastases progress to RAI-nonavid disease, similar to our case. Such cases present a treatment challenge. Within the last decade, the US Food and Drug Administration (FDA) approved tyrosine kinase inhibitors (TKIs), sorafenib and lenvatinib, for treating RAI-resistant progressive metastatic differentiated thyroid cancers (DTC). Patients treated with multi-targeted TKIs have shown progression-free survival in the DECISION[5] and SELECT[6] phase 3 clinical trials, albeit the overall survival benefit was not reached in these trials. However, these treatment options have undesirable side effects, which were experienced by > 95% of patients in both trials. We describe a unique case who presented with a confluence of adverse events (AEs) associated with the use of lenvatinib, including nephrotic syndrome, renal impairment, cardiomyopathy, and bicytopenia.

CASE PRESENTATION

Chief complaints

A 47-year-old female patient presented to our clinic in January 2001 with a swelling on the left side of her neck.

History of present illness

The patient noticed that the swelling gradually increased over the preceding six months without any pain or pressure symptoms.

History of past illness

She had a history of hypertension (HTN) and type 2 diabetes (T2DM) that were being treated with oral agents.

Physical examination

Physical examination revealed a non-tender swelling on the left side of her neck. It moved during the swallowing, was firm in consistency, and had no tethering of overlying skin. In addition, no palpable cervical lymph nodes were detected.

Laboratory examinations

Her complete blood count parameters as well as renal and thyroid function tests were normal.

Imaging examinations

An ultrasound scan of the neck revealed a large solid hypoechoic lesion with ill-defined margins measuring a maximum of 2.8 cm in the left lobe of the thyroid gland.

Further diagnostic work-up

Fine needle aspiration cytology showed follicular cells (many of which demonstrated a Hurthle cell change), raising the suspicion of Hurthle cell neoplasm.

FINAL DIAGNOSIS

She underwent total thyroidectomy in January 2001, and the procedure was uneventful. Histopathology showed Hurthle cell thyroid cancer (HCTC) with maximum tumor size of 2.5 cm. There was one focus of capsular and two foci of small vessel invasion, and the tumor extended to the perithyroidal soft tissue. One focus of micropapillary thyroid cancer, measuring 0.5 cm, was present in the left lobe without any invasion.

TREATMENT

She underwent diagnostic iodine 123 whole-body scan (WBS), which showed a tracer uptake of 1% in the thyroid bed. There was no uptake outside the neck. The stimulated thyroglobulin (TG) level upon thyroxine withdrawal was 723 mcg/L (reference 0-55 mcg/L) with thyrotropin stimulating hormone level of 59 mU/L (reference 0.35-5.5 mU/L) and free thyroid hormone (FT4) level of < 2 pmol/L (reference 11-23 pmol/L). Anti-TG antibodies were absent. She was administered a dose of 147 millicuries (mCi) radioiodine (RAI) ablation. Her post-therapy scans four days later did not show any new lesions.

In 2002, she had a recurrence in the neck, which was treated with bilateral neck dissection. Fourteen dissected lymph nodes were free of metastases. However, the pre-tracheal tissue deposit showed HCTC with prominent vascular invasion. This was followed by 60 Grays (Gy) of external beam radiotherapy to the neck delivered in two phases.

She continued to have an exceedingly high TG level, for which we performed diagnostic iodine 123 WBS that did not show any persistent or recurrent disease. In addition, 18F-fluorodeoxyglucose (FDG) positron emission tomography (PET) scan in 2003 showed no evidence of metastatic disease. In 2004, she underwent computed tomography (CT) scan of the chest, abdomen, and pelvis. CT revealed a solitary nodule (7 mm in size) in the middle lobe of the right lung, which remained stable on follow-up monitoring. Moreover, CT showed hepatic and bilateral renal cysts with radiological appearance consistent with polycystic kidney disease (PCKD). We administered another dose of 200 mCi RAI as empirical therapy in 2006 because of persistently elevated TG, although diagnostic iodine 123 WBS was negative. Her post-therapy scan did not show any radiotracer uptake.

OUTCOME AND FOLLOW-UP

She was continuously monitored, and in 2011, FDG PET scan showed FDG-avid right

renal mass measuring 4 cm in size and multiple micronodules in both the lung parenchyma (Figure 1). Iodine 123 WBS did not show any iodine-avid metastases (Figure 2). CT-guided biopsy of the renal mass revealed a renal oncocytoma. Given the benign nature of this renal tumor, she was monitored by urologists with no intervention. Lung nodules continued to increase in number and size, with some increasing to > 1.5-2.0 cm in size. Given this progression, in 2015, we suggested sorafenib. She reported that she felt healthy and elected not to pursue this option and was lost to follow-up. She revisited the clinic toward the end of 2017 when CT of the trunk showed significant progression of the pulmonary metastasis with some nodules > 2.5 cm in size (Figure 3). The renal mass also progressed in size, reaching 6 cm with evidence of invasion to the right renal vein. This etiology was inconsistent with a benign tumor; therefore, we reviewed the original biopsy from the renal mass. Immunohistochemistry was positive for thyroid transcription factor 1, paired box 8, and TG, which confirmed metastases from HCTC to the kidney.

The urology tumor board decided against performing a right nephrectomy because it would have left the patient with a solitary kidney with large cysts. Because of existing T2DM and HTN, there was a significant risk of future renal decline requiring renal replacement therapy; hence, no intervention was offered.

She was prescribed sorafenib 400 mg twice a day in March 2018 and she tolerated it well with a transient mouth ulcer. The dose was briefly reduced to 200 mg twice a day until the ulcer subsided. Surveillance radiological imaging at 3 mo and 6 mo showed disease stability without a reduction in TG levels. At 12 mo, PET showed progression in the number and size of pulmonary metastases (Figure 4). In August 2019, we discontinued sorafenib because of disease progression and initiated lenvatinib 24 mg daily, which has been shown to improve progression-free survival even in patients who have previously progressed with another TKI[6]. She tolerated the medication well with occasional diarrhea. Radiological monitoring at 3 mo and 6 mo showed stability with some reduction in the size of pulmonary metastases. TG levels declined in tandem with radiological stability (Figures 5 and 6A).

The patient experienced asymptomatic bicytopenia with a decline in red blood cells and platelets 10 wk after initiating lenvatinib. Hematological markers, including Coombs test, haptoglobin, reticulocyte count, lactate dehydrogenase, and peripheral blood morphology, did not indicate hemolysis. We managed the condition as an AE of lenvatinib and reduced the dose to 14 mg, which led to an improvement in indices (Figure 6B and C).

Further monitoring showed increasing proteinuria and a new impairment in renal function; hence, treatment was discontinued for two weeks and restarted with a 10 mg daily dose in March 2020. In May 2020, she presented with a 3-d history of diarrhea and acute kidney injury. She was administered intravenous fluid replacement and symptomatic care during hospitalization. Her proteinuria progressed to the nephrotic range, reaching 6 g per 24 h, coupled with a decline in estimated glomerular filtration rate (eGFR) to < 30 mL/min per 1.73 m^2 (reference eGFR > 90 mL/min per 1.73 m^2). At this stage, we discontinued lenvatinib. Five months later, we noticed a trend toward improvement with proteinuria reducing to 2.4 g per 24 h, plasma albumin rising to normal levels of 32 g/dL (reference 28-46 g/L) , and serum creatinine decreasing from 258 μmol/L to 160 μmol/L (reference 64-115 μmol/L) (Figure 6D and E). We have maintained regular telephone and clinic follow-up, and the patient continues to show improvement in the hematological and renal parameters, although the lung metastasis has shown a slight increase in size. She desires to restart TKI once her metabolic derangements normalize.

DISCUSSION

Our patient had a long history of T2DM, HTN, and PCKD, all of which can cause varying degrees of compromise to renal function as well as lead to proteinuria. However, she had normal baseline serum urea, creatinine, and eGFR levels. She had microalbuminuria before the initiation of lenvatinib. Renal indices worsened upon the initiation of lenvatinib but improved upon discontinuation, thus providing a clue toward a causal relationship of lenvatinib with respect to renal parameters in our patient.

Our patient had multiple large bilateral renal cysts. Moreover, the right kidney had a large metastatic tumor deposit from the thyroid cancer. In this context, performing a renal biopsy to determine the cause of nephrotic syndrome risked further compromise to an already impaired renal function. It was therefore deemed unsafe and not

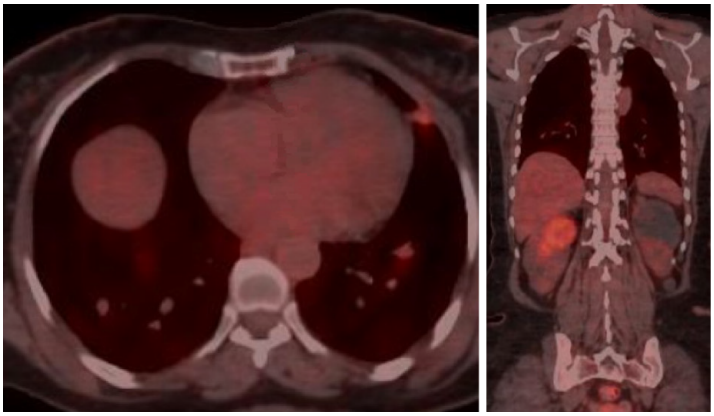


Figure 1 Positron emission tomography/computed tomography scan shows pulmonary metastases and new development of right renal metastasis.

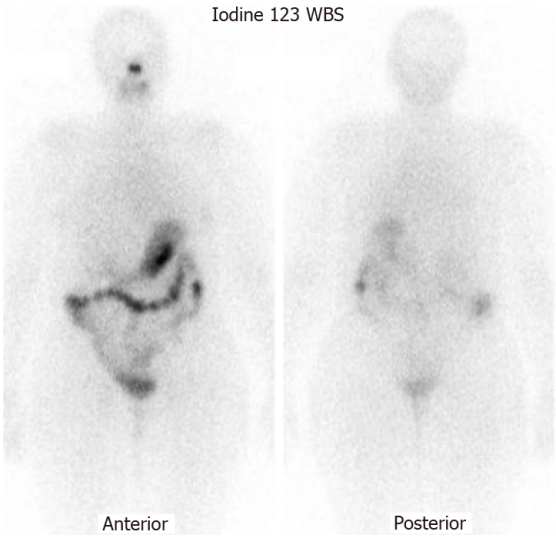


Figure 2 Diagnostic iodine-123 whole-body scan showing no tracer uptake in pulmonary and renal metastases. WBS: Whole-body scan.

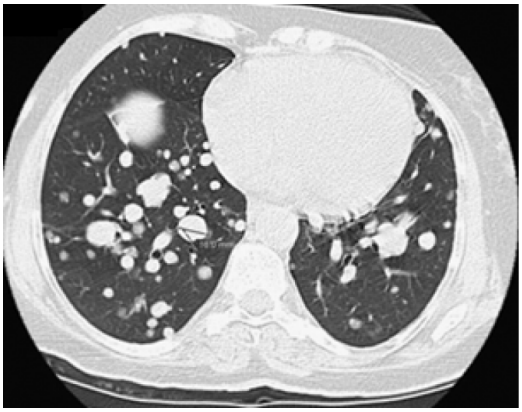


Figure 3 Chest computed tomography shows pulmonary metastases progression from Hurthle cell thyroid cancer.

performed.

Renal impairment is a well-recognized side effect associated with TKI use. Both sorafenib and lenvatinib are FDA-approved TKIs for use in patients with progressive metastatic RAI-resistant thyroid cancer. The DECISION trial did not report on sorafenib-associated renal adverse effects[5]. However, the incidence of sorafenib-associated proteinuria of any grade was 11.6%, whereas the incidence of grade ≥ 3 was

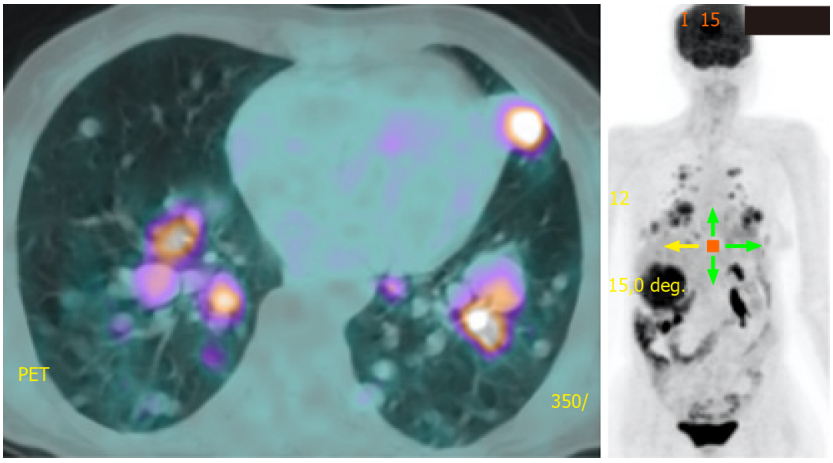


Figure 4 Positron emission tomography/computed tomography scan shows pulmonary metastases progression one year after treatment with sorafenib. PET: Positron emission tomography.

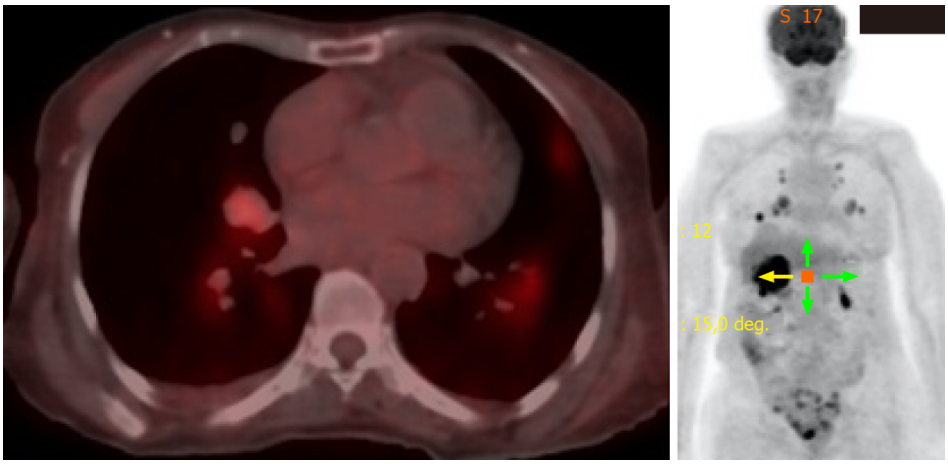


Figure 5 Positron emission tomography/computed tomography scan shows regression of pulmonary and renal metastases with lenvatinib.

0.9% in a recent meta-analysis[7].

In the SELECT trial involving lenvatinib, nearly one-third of all patients experienced some degree of proteinuria and 10% developed grade ≥ 3 proteinuria[6]. Some degree of renal impairment developed in 14% patients, while 1.9% developed grade ≥ 3 renal impairment. In a subgroup analysis of the Japanese population in the SELECT trial, the incidence of renal adverse effects was higher with any grade proteinuria of 63% and grade 3 proteinuria of 20%, even after adjusting the dose with weight[8].

The real-world experience of using lenvatinib and sorafenib in the Japanese population showed a much higher incidence of proteinuria (all grades) of 60.8% and 27.8%, respectively[9]. The mean change in eGFR was a decrease of 6.75% with lenvatinib *vs* an increase of 5.9% with sorafenib[9]. The median duration of treatment was 14.9 mo with lenvatinib and 4.6 mo with sorafenib in this retrospective study, which accounts for a higher incidence of proteinuria with lenvatinib. The authors reported five patients who developed nephrotic range proteinuria based on semiquantitative analysis using urine dipstick measurement, unlike our patient who had 24-h urine collection for estimating proteinuria.

An Italian real-life observational study that included 94 patients with DTC treated with lenvatinib reported reduced AE rates with grade ≥ 3 HTN of 4.7% and proteinuria of 1.7%[10]. These results are better than those observed in the SELECT trial[6]. The likely explanation for these superior results could be increased awareness, monitoring, and treatment of HTN and proteinuria by clinicians or gradual increase in the dose. A higher rate of AE observed in Japanese studies[8,9] and a lower rate of AE observed due to lenvatinib in the Italian study[10] suggest that different populations are distinctively predisposed toward AE risks with the same drug. Therefore, it is prudent

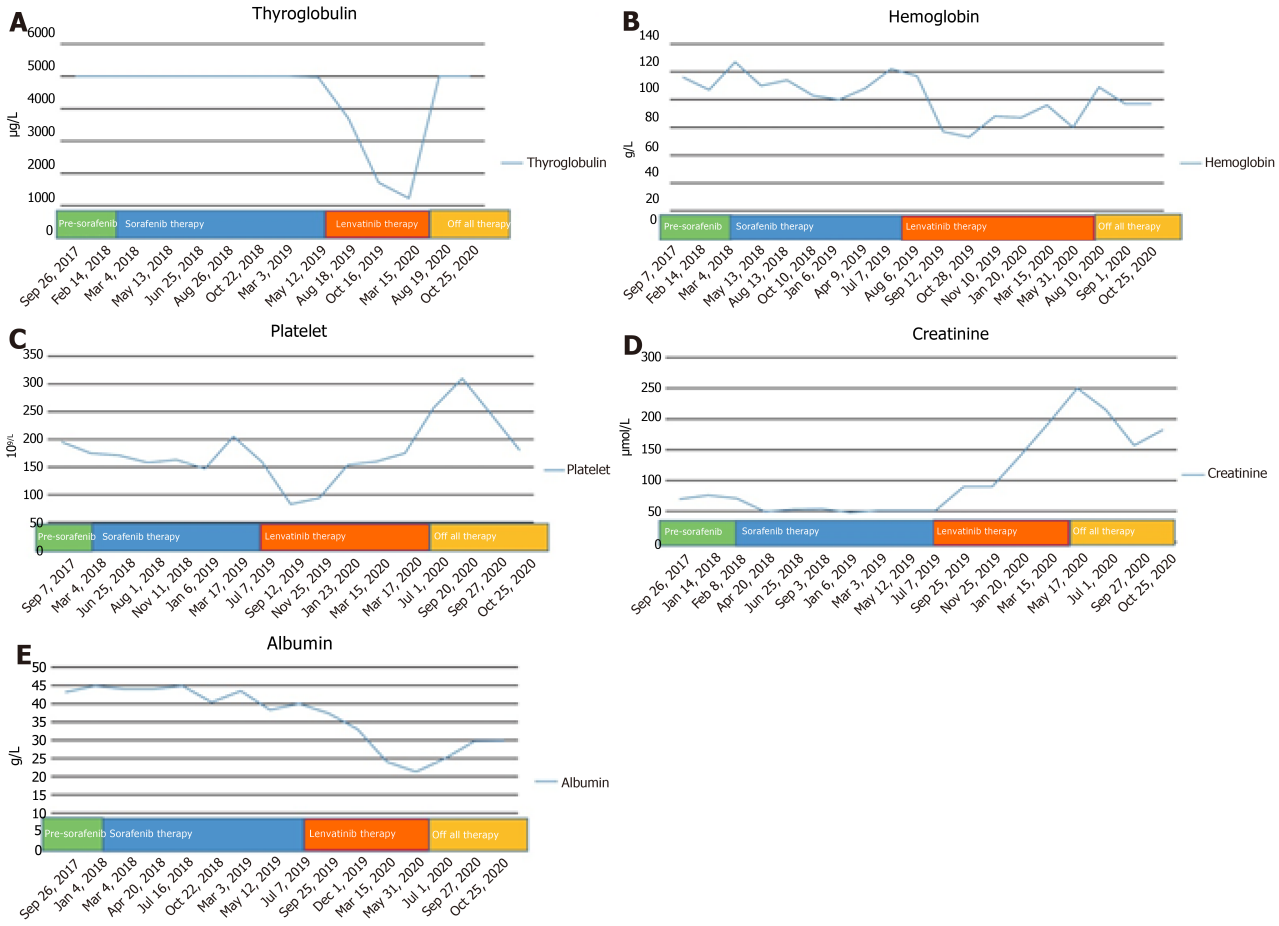


Figure 6 Blood test trends during tyrosine kinase inhibitor treatment. A: Thyroglobulin; B: Hemoglobin; C: Platelet counts; D: Renal profile; E: Serum albumin levels during tyrosine kinase inhibitor therapy.

to conduct an observational study in the Middle Eastern community to quantify the risk to our population.

A recent meta-analysis reported 18.7% of all grade and 2.4% of high-grade proteinuria with a nearly three-fold higher risk using anti-vascular endothelial growth factor (VEGF) TKI therapy[7]. The risk remained the same regardless of the renal or nonrenal site of the underlying cancer. Moreover, the risk varied depending on the use of different TKIs. However, the meta-analysis did not include any studies with lenvatinib. By contrast, Boursiquot *et al*[11] studied the use of anti-VEGF TKI therapy in patients with renal cell carcinoma. They found that it did not negatively impact the renal function of patients in the long term, even when used in patients with a pre-existing renal impairment or new development of HTN. They did not assess proteinuria pretreatment; however, only 7% of the patients had clinically relevant proteinuria during treatment.

Understanding the structure and normal function of glomeruli provides insight into the mechanism of TKI-induced renal injury. The fenestrated endothelium, the glomerular basement membrane, and the podocytes in the Bowman capsule form a physical barrier against the filtration of macromolecules in the glomeruli. The podocytes produce nephrin, podocin, and VEGF proteins that are essential for the development, maintenance, and function of the vascular endothelium within the glomeruli[12,13]. Anti-VEGF TKI therapies suppress the production of these proteins, which impair the normal morphology and function of glomeruli, causing glomerular injury that leads to renal impairment and proteinuria.

Treatment with anti-VEGF TKI therapies can lead to a new development or worsening of established HTN. Our patient had pre-existing HTN, and we optimized her medications as per requirement. There is some evidence that glomerular injury precedes the new development of HTN; therefore, HTN cannot be the sole trigger for proteinuria[12]. Moreover, some patients remain normotensive, yet they develop proteinuria. By contrast, some patients develop HTN without any accompanying proteinuria. This observation suggests that other factors may contribute to HTN

development. Horowitz *et al*[14] demonstrated that anti-VEGF therapies could block the VEGF-mediated vasodilation *via* the activation of nitric oxide (NO) synthase within the endothelial cells. Therefore, HTN may be observed in patients on anti-VEGF TKI therapy. A more recent study evaluated lenvatinib-treated patients with DTC, all of whom developed new HTN. They reported a drop in serum NO levels coupled with an increase in VEGF levels 6 d after treatment, suggesting that HTN may be caused by a reduction in NO levels at the level of vascular endothelium due to anti-VEGF therapy[15]. HTN development is considered as a surrogate marker of the therapeutic effect of anti-VEGF therapy, and those who develop new or worsening HTN show a trend toward survival benefit[11].

These targeted TKIs are well known to cause cardiovascular toxicity[16]. This study also carried out a meta-analysis, which included only sorafenib among the FDA-approved therapies for thyroid cancer, showed that it increased the risk of myocardial infarction, HTN, and left ventricular systolic dysfunction[16]. Moreover, both the DECISION[5] and SELECT trials[6] reported cardiac dysfunction presenting as the prolongation of corrected QT as well as impairment of cardiac function. Our patient had a normal echocardiogram during sorafenib treatment. However, she developed mild to moderate left ventricular systolic and diastolic dysfunction and ejection fraction reduction to 40%-45% during lenvatinib treatment. The 12-lead electrocardiograph did not show any abnormality.

Jensen *et al*[17] demonstrated that sorafenib-treated mice experienced deleterious effects in the myocardial metabolic pathways leading to systolic cardiac dysfunction, as shown on their echocardiograms after just two weeks of exposure. They noted a significant reduction in the levels of various metabolites that are involved in the taurine and hypotaurine metabolism pathways in the myocardium. Taurine, an amino acid, is available in abundance within the myocardium that functions in reducing injury caused due to oxidants[18]. Moreover, Ramila *et al*[19] found that taurine-deficient hearts had reduced sarcoplasmic reticulum calcium ATPase activity that affected the calcium sensitivity of myofibril proteins. They concluded that this could be responsible for deleterious effects on myocardial contractility.

Hematological derangements are not uncommon with TKI use. Our patient developed transient anemia and thrombocytopenia. Thrombocytopenia was the most common grade ≥ 3 AE observed in 25% of the patients in the meta-analysis of lenvatinib trials[20]. This observation was in sharp contrast with the 1.5% rate of grade ≥ 3 thrombocytopenia observed in the SELECT trial[6]. These variations could be due to different populations, inclusion/exclusion criteria, durations of exposure, pre-existing comorbidities, and concomitant use of other drugs that could predispose patients to these AEs.

VEGF receptor is present on most hematopoietic cells and peripheral cells, including the platelets and monocytes. The inhibitory effect of these anti-VEGF TKIs on hematopoiesis and myelopoiesis probably causes cytopenia. In addition, these multikinases block the platelet-derived growth factor receptor present on the hematopoietic stem cells and megakaryocytes. This hampers the function of platelet-derived growth factor, which promotes the production of platelets by direct effects on the bone marrow[21]. There is some evidence that thrombocytopenia could be caused by drug-induced immune-mediated response, as shown by the presence of antiplatelet antibodies during TKI treatment[22].

Kidneys are an exceedingly rare metastatic site from HCTC with only two reported cases in literature[23,24]. Both patients had iodine-avid metastasis; one patient underwent nephrectomy and RAI ablation cleared metastases in the other patient. Neither of these cases required treatment with TKIs. Our patient's cancer was noniodine avid and surgically unresectable, and she had PCKD. She required TKIs but experienced significant multiorgan AEs, all of which added more knowledge to the existing information regarding the treatment of such patients. Another unusual aspect of our patient was a progressive and metastatic tumor with a relatively low-risk pathology that otherwise carried a low risk of recurrence and metastasis[25].

CONCLUSION

In conclusion, the present case was unique and provided a unique learning and case management experience. The patient had widespread metastases despite a low-risk pathology, prolonged overall survival approaching two decades despite noniodine-avid distant metastases, and a confluence of multiorgan AEs with the use of lenvatinib.

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CASE REPORT

Tongue swelling as a manifestation of tongue metastasis from pulmonary sarcomatoid carcinoma: A case report

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Abstract

BACKGROUND

Sarcomatoid carcinoma is a rare subtype of non-small-cell lung cancer, commonly associated with locally advanced disease, early metastasis, and poor prognosis. Tongue metastasis from lung cancer is a rare condition that may occur in advanced stage of the disease.

CASE SUMMARY

The patient was a 70-year-old female with a history of resected pulmonary sarcomatoid carcinoma (PSC) who presented with subacute tongue swelling, imparting the clinical impression of a lingual abscess. However, histologic examination of the partial glossectomy revealed a high-grade, poorly differentiated spindle and epithelioid carcinoma consistent with metastatic PSC.

CONCLUSION

Although uncommon, clinicians should be cognizant of the possibility of a metastatic process to the tongue mimicking a benign or inflammatory process. A high index of suspicion for metastatic disease should be maintained when tongue swelling is observed in patients with a known history of PSC.

Key Words: Sarcomatoid carcinoma; Lung cancer; Tongue; Metastasis; Tongue swelling; Case report

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Core Tip: Tongue metastasis from pulmonary sarcomatoid carcinoma is an uncommon

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phenomenon. A high index of suspicion for metastatic disease should be maintained in patients with a known history of pulmonary sarcomatoid carcinoma when tongue swelling or radiologic image indicating a lesion in the tongue, especially near the tongue base, is observed, and appropriate investigations should be undertaken.

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INTRODUCTION

Sarcomatoid carcinoma is a rare biphasic tumor expressing both malignant mesenchymal (sarcomatoid) and epithelial (carcinomatous) components[1]. Pulmonary sarcomatoid carcinoma (PSC) is a poorly differentiated subtype of non-small-cell lung cancer (NSCLC)[2], commonly associated with aggressive clinical behaviors such as locally advanced disease, early metastasis, and poor prognosis. Tongue metastases are rare clinical events that may occur in advanced stage of the disease[3].

We herein report a rare case of tongue metastasis from a pleomorphic carcinoma, a subtype of PSC. To the best of our knowledge, this is the first documented case of tongue metastasis by this subtype of PSC.

CASE PRESENTATION

Chief complaints

A 70-year-old female presented with a 2-wk-history of tongue swelling and altered mental status.

History of present illness

Six months prior to admission, the patient underwent left upper lung lobectomy for pulmonary pleomorphic carcinoma, which is considered a subtype of PSC. The neoplasm was positive for mesenchymal-epithelial transition (MET) exon 14 skipping and *PIK3CA* mutations, with a programmed death-ligand 1 (PD-L1) positivity of 75%. The disease was staged as T4N1M0. She was started with capmatinib for stage IV PSC with MET exon 14 skipping mutation.

One month prior, she underwent left vocal cord injection for left vocal cord paralysis. Two weeks prior, she developed tongue swelling, dysphagia and odynophagia. A computed tomography (CT) of the neck with contrast was grossly unremarkable (Figure 1A), although visualization was limited due to dental streak artifact and motion artifact. The patient was started with methylprednisolone (Medrol) dose pack by her oncologist with capmatinib on hold as it was concerned that her tongue swelling could be an allergic reaction to capmatinib. However, her tongue swelling continued to worsen to the point that she was unable to take anything by mouth. She became more lethargic that prompted her presentation to the emergency department.

History of past illness

She had a prior medical history of atrial fibrillation, deep venous thrombosis, and bilateral hip osteoarthritis.

Personal and family history

She was a former 25 pack/year smoker, but had stopped smoking 25 years prior to this presentation.

Physical examination

On arrival, patient's peripheral capillary oxygen saturation (SpO_2) was 84% on room air. Physical examination revealed that the tongue and floor of the mouth were

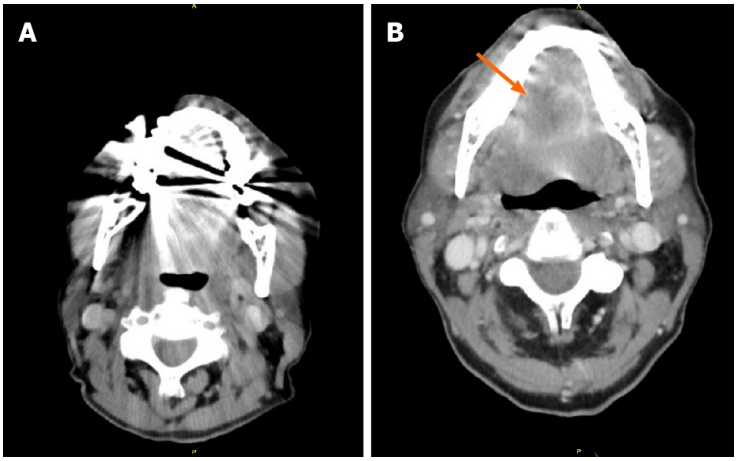


Figure 1 Computed tomography of neck. A: Computed tomography of neck visualization significantly limited due to motion and dental artifact; B: Pulmonary sarcomatoid carcinoma metastasis to tongue. A partially visualized peripherally irregular lesion measuring 2.7 cm × 3.2 cm × 1.9 cm within the tongue (arrow), with peripherally enhancement with central hypoattenuation, only partially visualized due to streak artifact from dental amalgam.

severely edematous; the deep oral tongue felt firm to palpation; the overlying mucosa was intact and of normal color; the submental space was mildly full; and cervical lymphadenopathy was not palpable.

Laboratory examinations

Laboratory studies revealed polymorphonuclear leukocytosis with white blood cell count of 20.85 × 10^3/μL.

Imaging examinations

Repeated CT of the neck with contrast (Figure 1B) identified a 2.7 cm × 3.2 cm × 1.9 cm lesion, which were not clearly visible in the CT one week earlier.

FINAL DIAGNOSIS

Histologic examination of the tongue mass showed a high-grade, poorly differentiated spindle and epithelioid neoplasm (Figure 2) that was positive for cytokeratin AE1/AE3 and TTF-1, but negative for p40. With positive staining for TTF-1, the tongue mass was considered a metastatic pleomorphic carcinoma of pulmonary origin to the tongue.

TREATMENT

Given the rapid interval change, an abscess was favored, with a differential diagnostic consideration of a necrotic mass. Patient was intubated for airway protection and admitted to the intensive care unit. She was started on empirical antibiotics with vancomycin and piperacillin/tazobactam. She then underwent midline partial glossectomy that revealed a submucosal mass without involvement of the tongue surface. It extended into the base of the tongue and the floor of the mouth.

OUTCOME AND FOLLOW-UP

The patient was extubated after the surgery with satisfactory oxygen saturation on room air and mentation back to normal. She then underwent percutaneous endoscopic gastrostomy tube placement for her persistent dysphagia. She was discharged home in a stable condition. To date, the patient has been receiving chemo-immunotherapy with carboplatin, Nab-paclitaxel, and pembrolizumab, with regular follow-ups with her oncologist.

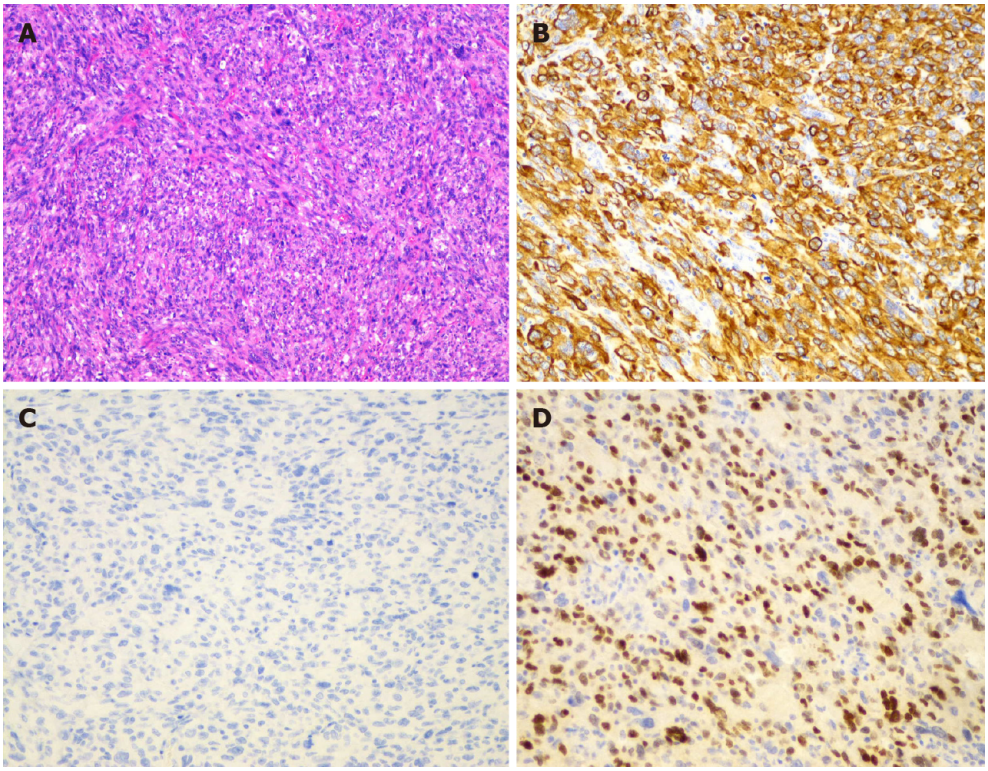


Figure 2 Histologic examination of the tongue mass. A: Tongue mass characterized by high-grade, poorly-differentiated spindle and epithelioid malignant neoplasm (magnification, 100 ×); B and C: Positive for cytokeratin AE1/AE3 (B) and TTF-1 (C), consistent with a metastatic carcinoma of pulmonary origin (magnification, 200 ×); D: The neoplastic cells are completely negative for p40 (magnification, 200 ×).

DISCUSSION

Sarcomatoid carcinoma is a biphasic tumor expressing both malignant mesenchymal (sarcomatoid) and epithelial (carcinomatous) components[1]. It can occur in various anatomical sites, most frequently in the head and neck, kidneys, and lungs. PSC is a very rare, poorly differentiated subtype of NSCLC, representing 0.1% to 0.4% of all lung malignancies[2]. According to the 2015 World Health Organization classification of lung tumors, PSC is sub-classified into five subtypes[4]: pleomorphic carcinoma, spindle cell carcinoma, giant cell carcinoma, carcinosarcoma, and pulmonary blastoma.

It is more common in men in their sixth decade of age[5]. It is linked to heavy smoking and asbestos exposure. Compared to conventional NSCLC, patients with PSC have shorter overall survival and higher recurrence rate. Distant metastasis appears more frequently than local relapse. Besides common metastatic sites for NSCLC, PSC also frequently metastasizes to the kidneys and gastrointestinal tract including the esophagus, small bowel, colon, and rectum[6].

Metastasizing to the oral cavity is uncommon, constituting 1%-3% of all oral malignancies[7]. Most metastases occur in the mandible, and oral soft tissue involvement is rare. The gingiva is the most frequent site for oral soft tissue metastases (57%), followed by the tongue (27%) and tonsils (8%). The most common cancers that metastasize to the oral cavity are tumors from the lung, breast, skin, gastrointestinal tract, liver, and male genitourinary tract[8]. Metastases of primary lung cancers to the tongue are rare, with an incidence varying between 0.2% to 1.6%[9]. In general, histologic analysis of primary lung cancers metastasizing to the oral cavity reveals adenocarcinoma or squamous-cell carcinoma.

Tongue metastasis usually present with a submucosal mass, commonly with intact overlying mucosa, but occasionally with ulceration or polypoid appearance. In one study[9], there were 12 cases of lingual metastases out of 5933 patients with different types of malignancies, with tongue base the most commonly affected site due to the relative immobility and rich vascular supply of lymphatics and blood. The tumor of our patient was submucosal, infiltrating the tongue muscles, and involving the tongue base, all of which are common features of metastasis.

Due to its rarity and lack of specific gross characteristics, at the time of detection, tongue metastasis may be mistakenly diagnosed as abscess, edema, or hemorrhage[8]. Common symptoms include pain, bleeding, discomfort, dysphagia, or dyspnea. Several imaging techniques, including ultrasonography, CT, and magnetic resonance imaging can be utilized for evaluation.

In our case, the patient presented with tongue swelling and associated dysphagia, odynophagia, and airway compromise. Clinically, it was initially considered an allergic reaction to her medication and later an abscess, while tongue metastasis was judged to be less likely. There were two factors that have compromised an initial accurate diagnosis. Firstly, the initial CT image could not be adequately viewed because of the artifacts from dental filling. The tongue lesion was only discovered on the following neck CT but not in the first one that was performed one week earlier. Secondly, the rapid progression of the disease, the presence of leukocytosis at presentation, and a history of recent invasive procedure to the adjacent anatomic area (injection for vocal cord paralysis), all of which place an acute pathologic process like abscess high on our list of differential diagnosis. Although clinical presentation with acute symptoms is uncommon for tongue tumors, it could occur due to necrosis or hemorrhage into the tumor. This case report provides two important lessons in patients presenting with space occupying lesions in unusual locations who already have pre-existing PSC. Firstly, accurate diagnosis by symptoms and image studies can be difficult, while pathology remains the most accurate diagnostic method. Secondly, the new lesion should be considered as the same pathologic process unless proven otherwise.

The literature on lung cancer metastasizing to the tongue is limited with only 12 cases reported from 1979 to 2020 (Table 1). Most primary pulmonary tumors were either squamous cell carcinoma or adenocarcinoma except for a single report of PSC. The patient was a 60-year-old man who had metastasis to the lateral border of the tongue two months after radical treatment for a spindle cell carcinoma, a subtype of PSC[10].

Surgical treatment of PSC is the main therapeutic strategy for resectable tumors. The effects of chemotherapy and radiotherapy are limited in advanced stages. MET mutation and PD-L1 overexpression are associated with a particular propensity for sarcomatoid carcinoma[21]. A higher frequency of MET exon 14 mutations has been reported in PSCs compared to other NSCLC, with a prevalence ranging between 22% and 32%[22,23]. Capmatinib is a highly selective MET inhibitor with antitumor activity in NSCLC. In May 2020, oral capmatinib received its first global approval in the United States for the treatment of metastatic NSCLC with MET exon 14 skipping mutation. Our patient was started on this new medication but failed to achieve a good response. PD-L1 is expressed in this type of tumors in 70%-90% of cases. There are several case reports demonstrating good response in PSC patients treated with immunotherapy[24,25], and our patient is now switched to combined chemo-immunotherapy.

CONCLUSION

Tongue metastasis from PSC is an uncommon phenomenon. To the best of our knowledge, the current case is the first case report in the literature of a primary pulmonary pleomorphic carcinoma metastasizing to the tongue and the second case report of tongue metastasis from PSC. A high index of suspicion for metastatic disease should be maintained in patients with a known history of PSC when tongue swelling or radiologic image indicating a lesion in the tongue, especially near the tongue base, is observed, and appropriate investigations should be undertaken.

Table 1 Studies on lung cancer with tongue metastasis

Ref.	Year	Basic information	Tongue metastasis as the initial presentation	Symptoms on presentation	Tongue metastasis location	Lung cancer pathology	Tongue as the sole metastatic site	Treatment	Outcome after diagnosis of tongue metastasis
Lee *et al*[11]	2018	63-yr-old; male; smoker	Yes	Dysphagia, painful tongue mass	Tip	Squamous cell carcinoma	Yes	Chemotherapy, tongue mass excision	Death after 8 mo
Cheng et al.[12]	2017	56-yr-old; male; smoker	No	Painful tongue nodule without ulceration, hoarseness	Base	Squamous cell carcinoma	Yes	Chemotherapy	Death after 7 mo
Jeba *et al*[13]	2016	45-yr-old; male; smoker	Yes	Tongue swelling without ulceration, dysarthria, dysphagia, hoarseness	Left anterior two-third, not crossing midline	Adenocarcinoma	No	Chemotherapy	Lost to follow-up
Ganesh *et al*[10]	2014	60-yr-old; male; unknown smoking history	No	Tongue swelling, left earache	Left lateral border	Spindle cell carcinoma (sarcomatoid carcinoma)	Yes	Patient refused treatment	NA
Mavili *et al*[8]	2010	58-yr-old; female; unknown smoking history	Yes	Painful tongue swelling, dyspnea	Right anterior	Adenocarcinoma	Yes	NA	Death after 2 mo
Wu *et al*[14]	2009	36-yr-old; female; unknown smoking history	No	Dysarthria, tongue swelling with deviation to left	Base	Adenocarcinoma	No	Chemo/target therapy and radiation	Death after 3 mo
Hatoum *et al*[15]	2008	63-yr-old; female; smoker	Yes	Painful tongue mass without ulceration, dysarthria, dysphagia	Entire right side, not involving the base	Adenocarcinoma	Yes	Chemotherapy	NA
Kurt *et al*[16]	2006	57-yr-old; male; smoker	No	Painful tongue nodule without ulceration	Anterior	Squamous cell carcinoma	No	Chemotherapy	Lost to follow-up
Terashima *et al*[17]	2004	63-yr-old; male; smoker	Yes	Painful tongue nodule without ulceration, dysarthria, dysphagia	Left side involving base and midline	Squamous cell carcinoma	No	Chemo-radiation	Death after 5 mo
Mui *et al*[18]	1999	65-yr-old; male; smoker	Yes	Tongue foreign body sensation and pain with ulceration, dysphagia, intermittent blood-streaked saliva	Right posterolateral	Large cell carcinoma	No	Chemo-radiation	Death after 4 mo
Shehab *et al*[19]	1994	70-yr-old; female; smoker	No	Tongue mass with ulceration	Right posterior one-third	Squamous cell carcinoma	No	Radiation	Death after 28 mo
Kim *et al*[20]	1979	74-yr-old; male; unknown smoking history	Yes	Tongue mass	Left anterior two-third	Adenocarcinoma	Yes	Radiation	Death during treatment

NA: Not available.

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REVIEW

Cellular based treatment modalities for unresectable hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and is unfortunately associated with an overall poor prognosis and high mortality. Early and intermediate stages of HCC allow for treatment with surgical resection, ablation and even liver transplantation, however disease progression warrants conventional systemic therapy. For years treatment options were limited to molecular-targeting medications, of which sorafenib remains the standard of care. The recent development and success of immune checkpoint inhibitors has proven to be a breakthrough in the treatment of HCC, but there is an urgent need for the development of further novel therapeutic treatments that prolong overall survival and minimize recurrence. Current investigation is focused on adoptive cell therapy including chimeric antigen receptor-T cells (CAR-T cells), T cell receptor (TCR) engineered T cells, dendritic cells, natural killer cells, and tumor infiltrating lymphocyte cells, which have shown remarkable success in the treatment of hematological and solid tumor malignancies. In this review we briefly introduce readers to the currently approved systemic treatment options and present clinical and experimental evidence of HCC immunotherapeutic treatments that will hopefully one day allow for revolutionary change in the treatment modalities used for unresectable HCC. We also provide an up-to-date compilation of ongoing clinical trials investigating CAR-T cells, TCR engineered T cells, cancer vaccines and oncolytic viruses, while discussing strategies that can help overcome commonly faced challenges when utilizing cellular based treatments.

Key Words: Hepatocellular carcinoma; Immunotherapy; Immune cells; Adoptive T cell therapy; Chimeric antigen receptor-T cell; Clinical trials

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Core Tip: Over the recent years investigation for safe and effective treatments for unresectable hepatocellular carcinoma (HCC) has shifted focus from various chemotherapeutic agents to immune based therapy. Although far from being finalized, immune cell-based therapy has shown efficacy in a variety of clinical trials, indicating possible future utilization alone or in combination for the prevention and treatment of HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a malignant primary tumor of the liver, accounting for approximately ninety percent of total liver tumor cases worldwide[1]. HCC commonly occurs in patients with preexisting liver cirrhosis, chronic hepatitis B virus (HBV), chronic hepatitis C virus (HCV), heavy alcohol consumption, aflatoxin exposure and metabolic associated fatty liver disease associated with diabetes and metabolic syndrome[2]. According to the Global Cancer Observatory (GLOBOCAN) in 2020, HCC is considered the 6th most common cancer worldwide, with the highest incidence in eastern Asia and Africa[3]. It has been estimated that by 2025, there will be more than one million individuals diagnosed with liver cancer annually[4]. Worldwide incidence of HCC has shown a greater predominance among men in comparison to women, with most diagnoses made in patients over the age of 60 years old[5]. HCC risk factors in the United States have varied significantly over the last two decades, with a majority of cases currently related to chronic HCV infection[6].

Management of HCC varies depending on the staging status of the tumor according to the standardized Barcelona-Clinic Liver Cancer (BCLC) system endorsed by both the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases[7]. Locally diagnosed tumors (BCLC stages 0-A), which are small in size (≤ 3 cm), early in progression, and limited to the liver parenchyma are amendable to surgical resection, radiofrequency ablation or ideally liver transplantation[8], with a median survival rate of 60 mo[9]. Advanced HCC on the other hand is defined by a significant increase in tumor size, vascular invasion and/or metastatic disease that is not amenable to loco-regional therapy (BCLC Stage C-D)[10], warranting treatment with systemic therapy[11]. In cases necessitating liver transplantation, the Milan criteria has been adopted by The United Network for Organ Sharing in order to determine eligibility[12], however this approach is limited by organ availability[13].

For over a decade, sorafenib has been considered the first line systemic treatment for advanced HCC. More recently, other systemic treatments such as the tyrosine kinase inhibitors (lenvatinib, regorafenib, and cabozantinib), immune checkpoint inhibitors (ICIs) (atezolizumab, ipilimumab, nivolumab, pembrolizumab) and the monoclonal antibody ramucirumab have been approved as first- and second-line treatment options[14]. With only modest established improvement in overall survival (OS) and an assortment of adverse effects, there has been a recent push for the development of immunobiological treatments, which first demonstrated efficacy in the treatment of hematological cancers. Treatments include chimeric antigen receptor-T cells (CAR-T cells), T cell receptor (TCR) engineered T cells, dendritic cells (DC), natural killer (NK) cells, and tumor infiltrating lymphocyte (TIL) cellular therapies (Figure 1). In this article we will review the novel range of cellular based treatments for HCC non-amenable to loco-regional therapy and introduce readers to various preclinical and clinical trials investigating their efficacy.

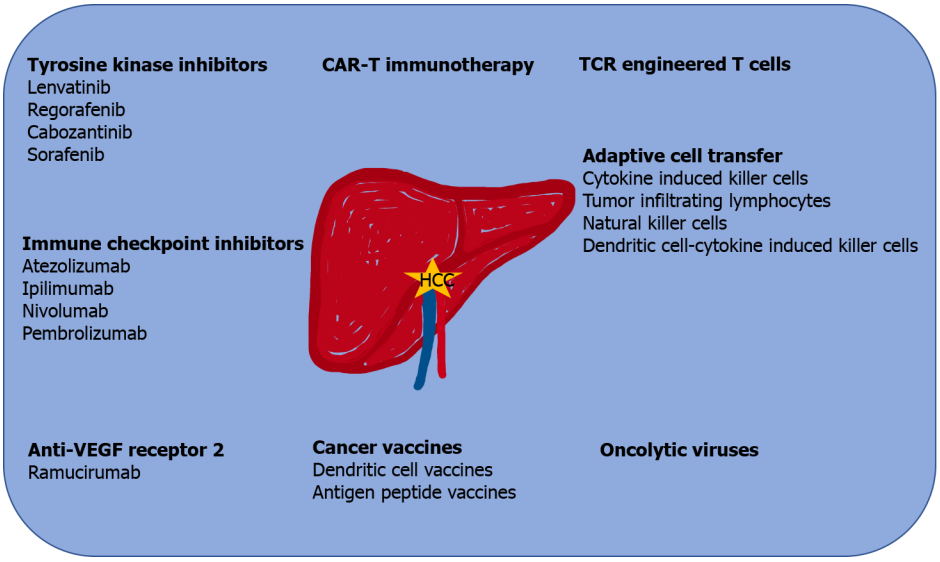


Figure 1 Treatment modalities available for unresectable (advanced) hepatocellular carcinoma, which is non-amendable to loco-regional therapies. CAR: Chimeric antigen receptor; HCC: Hepatocellular carcinoma; TCR: T cell receptor; VEGF: Vascular endothelial growth factor.

SYSTEMIC THERAPIES: APPROVED FIRST LINE THERAPIES

Sorafenib

Sorafenib is a multi-kinase inhibitor that inhibits cellular proliferation and angiogenesis through its effects on various receptor tyrosine kinases including vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and c-kit [15]. Its efficacy was demonstrated in two large phase III randomized controlled trials; SHARP conducted in the United States and Europe, and a similar study conducted in the Asia- Pacific focusing on patients with unresectable and advanced HCC[16]. In the SHARP trial, median OS was increased to 10.7 mo compared to 7.9 mo in placebo[17]. Similar findings were demonstrated in the Asia-Pacific trial; median OS increased in the treatment group (6.5 mo *vs* 4.2 mo) and time-to-progression (TTP) was also significantly greater (2.8 mo *vs* 1.4 mo)[18]. These findings led to sorafenib being approved by the Food and Drug Administration (FDA) in 2007, making it the standard of care and first line treatment option of advanced HCC[15,19].

Lenvatinib

Lenvatinib is another inhibitor of multiple receptor tyrosine kinases that has recently gained much attention[20]. In the randomized, open-label, non-inferiority phase III trial (REFLECT), lenvatinib was compared to sorafenib in patients who were deemed to have non-resectable HCC. Findings indicated an improved median OS of 13.6 mo with lenvatinib, compared to 12.3 mo with sorafenib. Lenvatinib also demonstrated an increase in median progression-free survival (PFS) and overall response rate (ORR) [21]. This allowed for FDA approval in 2018 as a fist line agent for treatment of unresectable HCC[22].

SYSTEMIC THERAPIES: APPROVED SECOND LINE THERAPIES

Ramucirumab

The phase III randomized controlled trial REACH (NCT01140347) investigated ramucirumab, a monoclonal antibody against VEGF receptor 2. While the study failed to demonstrate prolonged OS in the entire cohort, patients with elevated serum alpha-fetoprotein (AFP) (> 400 ng/mL) had significantly longer median OS when compared to placebo (7.8 mo *vs* 4.2 mo, P = 0.006)[23]. A subsequent phase III study, REACH-2 (NCT02435433) reported a significantly prolonged median OS (8.5 mo *vs* 7.3 mo) in sorafenib-experienced patients receiving ramucirumab *vs* placebo with AFP of 400 ng/mL or greater[24]. These findings showed promise as an effective treatment option in those with elevated AFP, leading to FDA approval in May 2019.

Regorafenib

Regorafenib is a multikinase inhibitor that inhibits various molecules including VEGF receptor 2/3, PDGF receptor and fibroblast growth factor receptor 1[25]. In the randomized, double-blind, phase III clinical trial RESORCE (NCT01774344) patients who failed sorafenib therapy were assigned to receive regorafenib *vs* placebo. This study determined that regorafenib significantly improved OS in individuals who experienced radiological progression of HCC during sorafenib treatment (10.6 mo *vs* 7.8 mo, $P < 0.0001$)[26]. Based on the promising findings of the RESORCE trial and a tolerable safety profile, the FDA approved regorafenib as a second line treatment option in those who have HCC progression while on sorafenib or who are not eligible for alternative therapy[25].

Cabozantinib

A double-blind, phase III clinical trial CELESTIAL (NCT01908426) was conducted testing cabozantinib, a multikinase inhibitor, in patients with unresectable HCC who had progressed with sorafenib. The study demonstrated a significant prolongation of OS when compared to placebo (10.2 mo *vs* 8.0 mo, $P = 0.005$) coupled with a longer median PFS when compared to placebo[27]. Based on the findings of CELESTIAL, the FDA approved cabozantinib as a second line treatment option for HCC in those who have undergone previous therapy with sorafenib.

IMMUNOTHERAPY: ICIS

Limited efficacy and undesirable side effects of traditional therapies (*i.e.*, sorafenib) have led to further investigation of immunotherapeutic agents over the years. A recent and emerging field of treatment includes cancer immunotherapies using ICIs that target programmed cell death protein-1 and its ligand (PD-1/PD-L1) and cytotoxic T-lymphocyte-associated protein- 4 (CTLA-4), which has significantly altered treatment of various types of cancer including HCC[28].

Immune checkpoint molecules play essential roles in regulating cancer cell interactions with the host immune system; and the development of drugs that suppress these interactions used by tumor cells to evade host immunity has shown promising results. PD-1, a member of CD28, is expressed on activated immune cells including T cells, B cells, and myeloid cells; and its interaction with ligand PD-L1 negatively regulates the immune system through suppressive signals that induce peripheral tolerance[29]. In HCC tumor cells, PD-L1 is aberrantly expressed, allowing for such an escape from host immunity[30]. CTLA-4 is another member of the CD28 family, and it is induced on T cells by antigen activation leading to subsequent binding with CD80 and CD86 with more affinity compared to CD28, negatively regulating costimulatory T cell signals and allowing for peripheral tolerance[31]. Ultimately these immunotherapeutic drugs strategically suppress key checkpoints that may be used by tumor cells to evade the host immune system, hindering a hallmark for tumor progression.

Atezolizumab

Atezolizumab is a fully humanized monoclonal antibody targeting PD-L1 that has recently changed the treatment landscape of unresectable HCC. A phase-Ib study evaluating synergistic atezolizumab and bevacizumab (anti-VEGF antibody) in patients with previously untreated and unresectable HCC not only demonstrated an acceptable safety profile, but promising antitumor activity with ORR 36%, disease control rate (DCR) 71%, and a median PFS of 7.3 mo when compared to monotherapy with nivolumab or pembrolizumab[32]. These results led to IMbrave150 (NCT 03434379), an open-label, randomized phase III study assessing atezolizumab (anti-PDL1) in combination with bevacizumab (anti-VEGF antibody) compared to sorafenib in patients with untreated locally advanced or metastatic HCC. OS at 12 mo was significantly longer in the combination group when compared to sorafenib (67.2% *vs* 54.6%) and PFS was significantly longer in atezolizumab + bevacizumab *vs* sorafenib (6.8 mo *vs* 4.3 mo, hazard ratio for disease progression or death [0.59; 95% confidence interval (CI): 0.47-0.76, $P < 0.001$], leading to FDA approval in May 2020 for the treatment of unresectable or metastatic HCC who have not received prior systemic therapy[33]. Currently a phase III, multicenter study of atezolizumab plus bevacizumab *vs* active surveillance as adjuvant therapy in HCC at high risk of recurrence after surgical resection or ablation is recruiting (IMbrave050; NCT04102098)[34]. In addition, a phase III study evaluating the safety and efficacy of atezolizumab plus

levantinib or sorafenib *vs* levantinib or sorafenib alone in locally advanced or metastatic and/or unresectable HCC following prior treatment with combination atezolizumab and bevacizumab was recently posted in February 2021 (IMbrave251; NCT04770896).

Ipilimumab

Ipilimumab is a monoclonal antibody targeting CTLA-4, which was recently granted accelerated FDA approval in May 2020, when used in combination with nivolumab (anti-PD-1) for HCC that has previously been treated with sorafenib. Approval was based on a single arm of the phase I/II clinical trial CheckMate 040 (NCT01658878), in which patients were treated with nivolumab 3 mg/kg plus ipilimumab 3 mg/kg every 3 wk for 4 doses. In addition to a substantial reduction in tumor burden, the overall ORR was 32%, with median OS 22.8 mo while having manageable safety profiles[35]. A phase III trial of ipilimumab in combination with nivolumab *vs* sorafenib or levantinib as first line treatment in patients with advanced HCC is currently recruiting participants (CheckMate 9DW; NCT04039607). A variety of clinical trials evaluating ipilimumab in combination with nivolumab during various treatment phases are ongoing and include; prior to liver resection (PRIME-HCC; NCT03682276), as neoadjuvant therapy (NCT03510871), and in combination with trans-arterial chemoembolization (CheckMate 74W; NCT04340193).

Nivolumab

Nivolumab is an anti-PD-1 monoclonal antibody that has shown promising results, leading to FDA approval for second-line treatment of advanced HCC. The phase I/II clinical trial CheckMate 040 (NCT01658878) investigated nivolumab in sorafenib-naïve and sorafenib-experienced patients with intermediate-advanced HCC and Child-Pugh Stage A. During the dose-expansion phase, ORR was 20%, while DCR was 64% with a median progression free survival of 4.1 mo[36]. The subsequent phase III study CheckMate 459 (NCT2576509) evaluating nivolumab *vs* sorafenib as first line treatments for unresectable HCC failed to demonstrate statistical significance for the primary endpoint of OS[37]. Currently a phase III trial, CheckMate-9DX (NCT 03383458), is actively evaluating recurrence free survival (RFS) in those with HCC at high risk of recurrence after curative hepatic resection or ablation.

Pembrolizumab

Pembrolizumab is a humanized anti-PD-1 monoclonal antibody that has been investigated across a variety of malignancies[38]. The phase II open-label clinical trial KEYNOTE-224 (NCT02702414) studied the safety and efficacy of pembrolizumab in patient with advanced HCC after having failed treatment with sorafenib. Favorable findings included an ORR of 17%, DCR of 61%, along with PFS of 4.8 mo, median OS of 12.9 mo and TTP of 4.9 mo[39]. This promising data led to FDA approval in November 2018 as a second line treatment after sorafenib. A subsequent phase III double-blind, randomized control trial, KEYNOTE-240 (NCT02702401) compared pembrolizumab to placebo, demonstrated longer OS and PFS; however, these findings were not statistically significant and failed to meet primary endpoints[40]. There are currently two-phase III trials that are ongoing at the time of this publication; KEYNOTE-394 (NCT03062358) , evaluating pembrolizumab in Asian patients with systemically treated advanced HCC and KEYNOTE-937 (NCT03867084) as an adjuvant therapy in HCC after curative treatment[29].

Ultimately, cancer uses a variety of unique mechanisms to evade host immune response and to develop drug resistance in HCC. This has sparked much interest in combining treatment modalities, particularly combination ICIs and checkpoint inhibitors with other chemotherapeutics. HIMALAYA (NCT03298451) is an open-label, multi-center, phase III study assessing durvalumab (anti-PDL1) plus tremelimumab (anti-CTLA4) in patients with advanced HCC. Scientists and physicians alike are eagerly awaiting results of other ongoing phase III trials such as LEAP-002 (NCT03713593) evaluating pembrolizumab and levantinib in combination and COSMIC-312 (NCT03755791) investigating atezolizumab and cabozantinib synergistically.

BEYOND CHECKPOINT INHIBITORS

Cell based immunotherapy

In recent years cancer therapy has begun shifting focus from the aforementioned conventional therapies, to the use of immune cell therapies, which utilize the host immune system to target and treat cancer. One such avenue of therapy includes gene modified T cell therapy, particularly TCR engineered T cells and CAR-T cells, which have shown promise in various malignancies and continue to be tested in clinical trials targeting HCC. Below we will discuss the concept of gene modified T cell therapies, and other modalities of adoptive cell transfer while reporting the results of recent and ongoing clinical trials where applicable.

When engineering T-cell based immune cell therapies targeting HCC, identification of tumor-associated antigens (TAAs) that can allow for appropriately mediated immune response is critical. AFP is a glycoprotein composed of 591 amino acids, identified as the first oncofetal biomarker for patients with HCC allowing for quantitative estimation of tumor burden and response to therapy[41]. Elevated levels of AFP have been found in approximately 70% of patients diagnosed with HCC, however elevations can also be found in other pathological conditions including cirrhosis, various hepatic disorders, germ cell tumors, lung cancer, gastric cancer, and pancreatic cancer[42]. Pre-clinical models have demonstrated the potential of AFP in the development of cellular immunotherapies[43,44]. Glypican-3 (GPC-3) belongs to the transmembrane heparan sulfate proteoglycan family, that regulates cellular division and growth[45]. GPC-3 expression is elevated in HCC, and recent studies have demonstrated that elevated levels correlate with an overall worse prognosis[46]. Therefore GPC-3 has been investigated in pre-clinical studies as a target for adoptive cell immunotherapy[47,48]. Melanoma antigen gene proteins (MAGE) was first identified in melanoma patients and has been found to be almost exclusively expressed in a variety of cancer tissues. Aberrant expression of MAGE has been demonstrated to significantly correlate with clinical characteristics of HCC, however functions of the multitude of MAGE proteins have yet to be thoroughly understood [49]. New York esophageal squamous cell carcinoma (NY-ESO-1) is a member of the cancer testis antigen family expressed in a variety of cancer cells including HCC[50]. NY-ESO-1 has been deemed as the most promising cancer testis antigen for the development of cancer immunotherapy with a multitude of studies demonstrating promising results across a variety of malignancies[51]. When examining HCC caused by viral infection (HBV or HCV) there is prospect in targeting viral antigens as a strategy to etiologically treat HCC. Targets include HBV S or L protein (envelope proteins), which have shown promise by eliminating HBV positive hepatocytes when targeted by antigen specific T cells[52]. Targeting of hepatitis B surface antigen has also shown efficacy in mouse models utilizing CAR-T cell therapy as well[53]. Other tumor antigens that are of particular interest include epithelial cell adhesion molecules (EpCAM)[54], mucin 1 glycoprotein (MUC1)[55] and human telomerase reverse transcriptase (hTERT)[56].

CAR-T and TCR engineered T cells: The basics

Gene modified T cell therapy is a method used to deliver T cells that are genetically engineered to produce TCRs that recognize specific tumor associated antigens and their epitopes. There are currently two methods of developing gene modified T cells, CAR-T cells and TCR transgenic T-cells (engineered T-cells), which have both shown efficacy and promise in a variety of solid and hematological malignancies[57]. CAR is composed of three basic elements: (1) the extracellular antigen recognition domain, (2) the transmembrane domain and (3) the intracellular signaling domain. The extracellular portion is a single chain antigen recognition receptor composed of heavy and light chains of a monoclonal antibody specific to the tumor surface antigen, allowing for no restrictions of target antigen recognition by major histocompatibility complex (MHC) molecules. The intracellular portion is formed by combining co-stimulatory molecules to the intracellular portion of TCRs[58]. The basic process of CAR-T generation includes: (1) T cell isolation from peripheral blood mononuclear cells using immunoselective beads and activation with anti-CD3 and Il-2, (2) genetic modification of CAR-T, (3) expansion of T cells *in vitro*, (4) evaluation ensuring CAR expression and T cell viability, and (5) CAR-T infusion back into the patient[59]. TCR transgenic T cells possess a TCR on the surface consisting of two distinct protein chains; alpha and beta, which bind to the MHC of antigen presenting cells, allowing for a highly specific interaction which is not limited to membrane bound antigens[60,61]. As previously described the selection and validation of target antigens is of utmost importance when

designing both TCR-T cells and CAR-T cells that are efficacious and minimize on target/off-tumor side effects[62].

CAR-T immunotherapy

CAR-T cell therapy has been tested both preclinically and clinically when targeting HCC. Below we will discuss findings of completed and ongoing clinical studies that seek to demonstrate safety and efficacy of cellular therapy. Shi *et al*[63] recently published results from two prospective phase I trials involving GPC-CAR-T cells (NCT02395250 and NCT03146234) in patients with advanced GPC3 positive relapsed or refractory HCC following chemotherapeutic induced lymphodepletion. A total of 13 patients received CAR-T cell infusion, with two partial responses and one patient with sustained stable disease after 44.2 mo. OS rates at 3 years, 1 year, and 6 mo were 10.5%, 42% and 50.3% respectively with median OS duration of 278 d according to the Kaplan-Meier method. Unfortunately, toxic effects were noted with major concern as one patient developed cytokine release syndrome (CRS) leading to death from multi-organ failure[63]. A recently completed, open-label, phase I-II study by Dai *et al*[64] demonstrated efficacy of CD133-CAR-T cell therapy in patients with biopsy proven HCC (BCLC stage C) not amenable to curative treatment. One patient demonstrated a partial response, while 66.7% of patients had stable disease after infusion with an overall PFS of 6.8 mo and median OS of 12 mo (NCT02541370). Currently an open-label, single center, phase I-II study is underway investigating CAR-T/TCR-T cell immunotherapy targeting a variety of different malignancies including hepatoma (NCT03638206). A list of currently ongoing clinical trials investigating CAR-T cells for the treatment of HCC is listed in (Table 1).

Improving CAR-T delivery and efficacy

Proven clinical efficacy of CAR-T across a variety of hematological malignancies has led to the investigation of its use in solid tumors. However, treatment of solid tumors poses many challenges to clinical investigators, one of which includes access to the tumor site. Many cases of HCC occur on the background of a fibrosed and cirrhotic liver, and the presence of such fibrotic extracellular matrix (ECM) poses a barrier for CAR-T cell penetration. In order to overcome such a challenge, CAR-T cells co-expressing heparinase, which degrades ECM heparan sulfate proteoglycans were developed. These newly engineered CAR-T cells improved cellular ability to degrade the ECM and displayed antitumor activity, suggesting a feasible strategy for the development of CAR-T cells targeting stroma-rich solid tumors[65]. Similar strategies are being investigated with the use of matrix metalloproteinase 8, which can allow CAR-T cells to overcome physical barriers[66]. Local administration of immunotherapy directly into tumors has shown promising results for organ selective delivery. In a study conducted by Katz *et al*[67], not only were CAR-T cells detected in biopsies of liver metastasis, but effective CAR-T cellular response was noted within the tumor following delivery *via* hepatic artery infusion. Such results have paved light into the investigation of direct tumor inoculation *via* a variety of local administration techniques including hepatic artery infusion in a multitude of clinical trials. Novel delivery platforms including nanoparticles, scaffolds and other biomaterials are also being investigated as a safe and effective way to deliver immunotherapy to tumor cells while avoiding off-target adverse effects[68]. Recent findings from the 2020 study conducted by Ma *et al*[69], demonstrated superior targeting and anti-tumor capabilities of CAR-T cell membrane coated nanoparticles both *in vivo* and *in vitro*.

In addition to tumor location being inaccessible, the tumor microenvironment has been found to be detrimental to CAR-T cells in regard to their survival and proliferation. The consumption of glucose by malignant cells renders the surrounding environment hypoxic, acidic, and low in nutrients leading to oxidative stress; leaving glycolytic T cells unable to adequately function, ultimately hindering the immune response[70,71]. In order to help eliminate reactive oxygen species (ROS) accumulation, CAR-T cells co expressing catalase were designed, and were subsequently able to survive and function in such unfavorable environments[72]. However, oxidative stress with release of compounds such as ROS and hydrogen peroxide, not only prevents recruitment and adequate function/persistence of immune cells, it also allows for recruitment of immune suppressor cells that lead to CAR-T cell exhaustion [73]. Strategies to help overcome such exhaustion include the use of ICIs that target PD-1, PD-L1, CTLA-4 in conjunction to CAR-T cells administration[74], and by genetically deleting T-cell PD-1 protein with the use of CRISPR-CAS9 gene editing [75]. There is optimism that these findings coupled with continued advance-ments in biomedical engineering will allow for new delivery and tumor microenvironment altering capabilities that can help amplify immunotherapeutic response in the

Table 1 Ongoing clinical trials investigating chimeric antigen receptor-T cell therapy in the treatment of hepatocellular carcinoma according to ClinicalTrials.gov

Product	Phase	Estimated completion	Study/identifier
GPC3- CAR-T	I	November 2021	NCT04121273
CD147- CAR-T	I	May 2022	NCT03993743
GPC3- CAR-T	I	June 2022	NCT03980288
CAR-CLD18	N/A	December 2023	NCT03302403
GPC3- CAR-T	I	May 2024	NCT03884751
GPC3/TGF-CAR-T	I	August 2024	NCT03198546
GPC3- CAR-T	I	October 2036	NCT02905188

CAR: Chimeric antigen receptor; CD: Cluster of differentiation; GPC3: Glypican-3; TGF: Transforming growth factor.

treatment of HCC.

Improving CAR-T safety

When utilizing cellular immunotherapy with CAR-T cells, a number of important safety concerns are to be noted including CRS, central nervous system toxicity (CNS) and the "on target-off tumor" effect. CRS is characterized by flu like symptoms including fever, fatigue and headache, which can progress to hypotension and organ dysfunction secondary to uncontrolled systemic inflammatory response associated with elevated serum cytokines; particularly interleukin (IL)-6, IL-10, tumor necrosis factor α (TNF-α) and interferon (IFN)-γ[76]. Symptoms of CNS toxicity can range from headache and dizziness, to memory loss and delirium, and can be found along with CRS or after the resolution of CRS. Although the exact etiology remains unclear at this time, it is speculated that various cytokines may alter the blood brain barrier[77], in conjunction with an increase in protein, white blood cells, IFN-γ, TNF-α, and IL-6 within cerebrospinal fluid[78].

These adverse reactions stem from CAR-T cells recognizing TAAs, which are not only heterogenous amongst tumors, but can also be present in normal tissues, elevating the risk of off target toxicity[71]. Such toxic effects have been reported following administration of anti-ERBB2 CAR-T cells for the treatment of metastatic malignancy, which led to CRS with subsequent respiratory distress and death due to low levels of ectopic ERBB2 in lung epithelium[79]. In order to help overcome such toxicities, a variety of strategies are being investigated. One such strategy includes the controlled removal of CAR-T cells with the use of suicide genes. There has been promise with the use of a drug inducible caspase 9 system (pro-apoptotic molecule that activates downstream pro-apoptotic caspase 3 when chemically induced) leading to apoptosis of CAR-T cells on demand[80] and with a system utilizing a truncated epidermal growth factor receptor, which allows for elimination following administration of a receptor specific antibody[81]. More recently, Amatya *et al*[82] developed CAR-T cells with a caspase 9 suicide gene for the treatment of multiple myeloma. Following administration of a dimerizing agent, CAR-T cells underwent apoptosis and were no longer active.

As previously mentioned, identification of a suitable TAA is imperative in the development of efficacious CAR-T cells, however highly specific TAAs are rarely identified, leading to investigation of dual- targeted CAR-T cells. Chen *et al*[83] developed dual-target CAR-T cells (targeting GPC3 and Asialoglycoprotein receptor 1) for the treatment of HCC. Not only did these cells demonstrate superior anticancer activity coupled with higher cytokine secretion when compared to single target CAR-T cells, but their specificity toward cellular targets may reduce the risk of on target- off tumor toxicity[83]. Recent developments have shown efficacy of a novel, split anti-GPC3 CAR-T, into two components with the use of SpyCatcher (extracellular 116 amino acid sequence linked to the intracellular domain) and SpyTag (13 amino acid peptide linked to single chain variable fragment). Such dual component delivery of CAR-T cells not only suppressed tumor growth, but displayed decreased cytokine release, representing a safer alternative when compared with conventional CAR-T cells[84]. Other perspectives include formation of a split, universal and programmable CAR system that allows for enhancement of specificity, safety and programmability of

CAR delivery systems[85].

TCR engineered T cells

TCR engineered T cells are modified T cells that are able to precisely recognize tumor surface antigen peptides and MHC, rendering a response only effective when tumor cells express both antigen epitopes and MHC molecules[57]. TCR engineered T cells specific to NY-ESO-1 have demonstrated significant antitumor effects in the treatment of multiple myeloma[86] and synovial sarcomas[87]. The presence of NY-ESO-1 over-expression in HCC has made it an investigated target when developing engineered T cells in one phase II study (NCT01967823), and two phase I studies (NCT02869217) and (NCT03159585), for which we eagerly await results. In addition, TCR engineered T cells targeting hepatitis B viral antigens in HBV related HCC have been of interest in both preclinical and clinical models[88,89]. Recently in November 2020, Chen *et al*[90] presented an abstract at the 2020 Society for Immunotherapy of Cancer, demonstrating safety and efficacy of HBV specific TCR T-cells in recurrent HBV related HCC patients post liver transplant (NCT02719782).

There are currently five ongoing trials investigating TCR engineered cells targeting various TAAs in HCC (Table 2). All trials are investigating efficacy in advanced, unresectable HCC, with the exception of (NCT03899415), which is evaluating the safety and clinical benefit of TCR engineered T cells in HBV positive HCC patients post hepatectomy or radiofrequency ablation.

Cytokine induced killer cells

Cytokine induced killer (CIK) cell therapy is a form of adaptive cell transfer that has been investigated as adjuvant therapy in patients with HCC. CIK cells are isolated from the patient's peripheral mononuclear cells, at which point they are grown *ex vivo* with the addition of cytokines such as recombinant human IFN-γ, anti-CD3 monoclonal antibody, and recombinant human IL-2 prior to transfusion back into the patient; allowing for recognition and subsequent death of tumor cells[91]. In a multicenter, randomized phase III trial conducted in Korea, 230 patients were assigned to receive adjuvant CIK or no adjuvant therapy (control) following treatment with surgical resection, radiofrequency ablation or percutaneous ethanol injection (NCT00699816). Median RFS was significantly prolonged in the immunotherapy group (44 mo *vs* 30 mo, P = 0.01), coupled with lower hazard ratios for all cause death (P = 0.08) and cancer-related death (P = 0.02)[92]. A 5-year follow-up study demonstrated continued efficacy of autologous CIK immunotherapy without any repeated injections (NCT01890291). After an average 68.5 mo follow up, RFS was 44.8% in the immunotherapy group and 33.1% in the control group, coupled with a significantly lower risk of all-cause death (hazard ratio 0.33, 95%CI: 0.15-0.76, P = 0.006)[93]. Similar results were demonstrated in a meta-analysis and systemic review conducted by Cai *et al*[94] examining adjuvant CIK after invasive treatments for HCC. Pooled analysis demonstrated a significant improvement of RFS, PFS and OS in patients receiving autologous CIK immunotherapy. Although many studies have been conducted examining the safety and efficacy of CIK as an adjuvant treatment for HCC with mixed results; research is limited when examining CIK therapy as a sole treatment for unresectable HCC.

TILs

TIL therapy is an immunotherapeutic technique that involves isolation and subsequent cell culture followed by autologous administration of tumor specific T cells present in infiltrated tumors. Success of adoptive cell therapy using TIL is based on the diverse antigenic specificity displayed by TIL cells toward the tumor and the lytic capabilities possessed allowing for eradication of malignancy[95]. TILs play an essential role in cancer progression[96] and even as a predictive biomarker of response to neoadjuvant chemotherapy in a variety of malignancies[97]. After the recognition of tumor associated antigens[98], various types of TILs infiltrate to the tumor site; including NK cells, NK T cells, mucosal-associated invariant T cells, and gamma delta T cells[99]. CD4+ T helper cells on the other hand further differentiate in the periphery into a variety of subsets (*i.e.*, Th1, Th2, Th9, Th17, Th22, regulatory T cells, and T follicular helper cells)[100]. CD8+ and NK cells contribute to a direct cytotoxic antitumor effect induced by a cascade of activating and inactivating receptors[99].

In a study conducted by Chew *et al*[101], examining immune gene expression profiles of resected tumor, HCC patient survival was positively correlated to higher expression of inflammatory immune genes. In conjunction, there was an increased presence of NK cells and T cells promoting apoptosis and reducing proliferation in the

Table 2 Ongoing clinical trials investigating T cell receptor engineered T cells in the treatment of hepatocellular carcinoma according to ClinicalTrials.gov

Product/target	Phase	Estimated completion	Study/identifier
C-TCR055/AFP	I	April 2021	NCT03971747
C-TCR055/AFP	I	November 2021	NCT04368182
HBV-TCR T cell/HBV Ag	I	June 2024	NCT03899415
IMA202-101/MAGE A1	I	June 2024	NCT03441100
AFP T cells/AFP	I	June 2026	NCT03132792

AFP: Alpha fetoprotein; HBV: Hepatitis B virus; Ag: Antigen; MAGE: Melanoma antigen gene protein.

tumors of patients with longer survival, suggesting that TILs can be used as a specific immunotherapy for treatment of HCC[101]. In a hallmark randomized trial, patients who had undergone curative resection for HCC were assigned to receive adoptive immunotherapy *vs* no adjuvant therapy. Administration of lymphocyte infusion led to a reduced frequency of recurrence by 18%, significantly longer time to first recurrence (P = 0.008), longer RFS (P = 0.01) and disease- specific survival (P = 0.04) when compared to controls[102]. A phase I trial (NCT01462903) examined the use of autologous TILs in HCC patients post resection. Results demonstrated that 80% of patients remained disease free after 14-mo follow-up, while displaying a favorable side effect profile, further suggesting TIL therapy as a safe treatment method for HCC[103]. Currently an active clinical trial is investigating the safety and efficacy of autologous TILs in patients with high-risk recurrent HCC (NCT04538313). Research is limited on the use of TIL immunotherapy for unresectable HCC, and we hope that further studies can be initiated using TILs alone or in combination with other therapies such as chemotherapy or ICIs.

NK cells

NK cells play an active and critical role in the innate and adaptive immune defense against viral infections and hepatocellular malignancy[104], forming as much as 50% of innate immunity cell infiltrate within the liver[105]. Several clinical studies have demonstrated the safety and efficacy of allogenic NK cell adoptive immunotherapy in the treatment of various malignancies[106,107] including HCC[108]. In a study conducted by Lin *et al*[108], patients were enrolled to receive cryoablation alone *vs* allogenic NK cell therapy for the treatment of unresectable HCC. Results demonstrated synergistic effects of combination therapy leading to enhanced immune function, reduction in AFP, and improved quality of life in addition to an increased median PFS (9.1 mo *vs* 7.6 mo, P = 0.01), higher response rate (60% *vs* 46.1%, P < 0.05) and DCR (85.7% *vs* 69.2%, P < 0.01) when compared to cryoablation alone. Similar results were demonstrated in a study conducted by Alnaggar *et al*[109], which investigated allogenic NK cell immunotherapy in combination with irreversible electroporation for Stage IV unresectable HCC. Efficacy of treatment was synergistic, with statistically significant lower AFP levels at 1 and 3 mo after treatment (P < 0.01), coupled with a higher median OS (10.1 mo *vs* 8.9 mo, P = 0.0078) when comparing combo therapies *vs* electroporation alone. Currently a phase I/II study investigating allogenic NK cell therapy in patients with advanced HCC (NCT04162158) is underway, as well as a phase II/III study examining the effects of autologous NK therapy when coupled with transcatheter arterial chemoembolization (TACE) for the treatment of advanced HCC (NCT04011033). Combination therapy of "off the shelf" FT500 NK cells with ICIs is also being investigated in subjects with advanced solid tumors including HCC (NCT03841110).

The advent of genetic modification techniques has led to the use of CAR technology to develop CAR-NK cells to improve specificity and efficacy of NK cell cytotoxi-city [110]. Yu *et al*[111] developed a GPC3-specific NK cell for the treatment of HCC, which demonstrated *in vitro* cytotoxicity and cytokine production in addition to potent anti-tumor activities in HCC xenografts. Similar treatment benefits were noted with the development of c-MET specific CAR-NK cells, which demonstrated specific cytotoxicity against malignant hepatic cells *in vitro*[112]. While there are many ongoing clinical trials investigating CAR-NK immunotherapy for the treatment of hematological malignancies; investigation of treatment efficacy in solid tumors remains

scarce.

DC-CIK cells

DC are antigen presenting cells that bridge the gap between innate and adaptive immunity, allowing for activation of naïve T cells that promote tumor-specific T cell immune response and adaptive immunity[113]. The use of *in vitro* generated DC combined with CIK cells has shown to be an effective and promising immunotherapy technique when used alone or in combination with other treatment options for a variety of malignancies[114]. In a study conducted by Zhou *et al*[115], advanced HCC patients were assigned to receive sorafenib alone *vs* a combination of sorafenib and DC-CIK immunotherapy. Results not only demonstrated a significant reduction in AFP levels when using combination therapy, but a significant increase in clinical benefit rate (41.9% *vs* 88.6%, $P < 0.05$) and prolonged median survival time (13.8 m *vs* 18.6 m, $P < 0.05$) while maintaining safety. Examination of combined therapy of TACE with DC-CIK therapy *vs* TACE alone has shown statistically prolonged OS time in HCC patients with HBV[116]. A meta-analysis of 22 studies, conducted in 2019 by Cao *et al*[117], has shown a prolonged survival and reduced recurrence rate of HCC when combining conventional clinical treatment and immunotherapy with DC and/or CIKs. There are currently studies recruiting participants for treatment of various solid tumors (including HCC) with DC-CIK combination immunotherapy (NCT04214717, NCT04476641, NCT03190811).

HCC vaccines

Current advancements in medicine and immunobiology have demonstrated promising treatment strategies of cancer vaccines and oncolytic immunotherapy in patients with advanced stage cancer, leading to a variety of studies under clinical investigation [118]. Modalities include DC vaccinations, vaccines that target the antigens on tumor cells which in return activate cellular and humoral immunity causing a phagocytic activity towards the tumor cells, and oncolytic viruses that can attack and destroy tumor cells. These vaccines have both prophylactic and therapeutic aspects that can aid in the treatment of advanced cases of HCC. An up-to-date list of cancer vaccines and oncolytic virus therapies under current clinical investigation is provided (Table 3).

Antigen peptide vaccines

Targeting these unique antigens can contribute to a significant approach in the development of vaccines in cases of HCC. Foremost antigens include AFP, GPC3, NY-ESO-1, hTERT, and hepatocellular and melanoma antigen gene-A (*MAGE-A*) as previously mentioned[119]. GPC3 peptide, a heparan sulfate glycoprotein and member of the GPC proteoglycan group, functions through attaching to the cell membrane by a glycophosphatidylinositol anchor, further regulating several growth factors through the Wnts, hedgehog, and the BMP signaling pathways[120]. This peptide can be an ideal target for HCC vaccine development, as GPC3 is detected in the vast majority of HCC cases[121]. In a phase I trial conducted on 33 patients to ensure the safety of the GPC3-derived peptide vaccine for advanced HCC, results indicated a well-tolerated vaccine with significant immune response, and an improvement in the median OS rate, 12.2 mo (95%CI: 6.5-18.0) in patients with high GPC3-specific cytotoxic T-lymphocytes frequencies, as compared with 8.5 mo (95%CI: 3.7-13.1) in those with low GPC3-specific cytotoxic lymphocyte frequencies ($P = 0.033$)[122]. However, it was not determined if these tumor-infiltrating lymphocytes detected after vaccination were GPC3 peptide-specific cytotoxic lymphocytes, prompting the initiation of a phase II trial (UMIN000005093) in patients with advanced HCC having failed sorafenib, which demonstrated induction of GPC3 peptide specific cytotoxic lymphocytes capable of infiltrating tumors[123].

DC vaccines

DCs have a potent effect on the T cell responses in HCC and other malignancies by acting as antigen presenting cells towards cytotoxic T lymphocytes[124]. Prior to administration of these vaccines to subjects, allogenic DCs are loaded with tumor antigens *ex vivo*, and then stimulated with cytokines such as granulocyte-macrophage colony-stimulating factor for further expansion[125]. In a recent 2016 study, DC vaccines loaded with autologous tumor cell lysate (Hepa1-6 cells) were studied using an orthotopic murine model of HCC. Treatment using this combination not only inhibited progression of murine HCC, but results were promising with a 90% survival rate compared to survival rate lower than 5% in untreated mice, suggesting alteration of the immunosuppressive tumor microenvironment as measured by lymphocyte and

Table 3 Ongoing clinical trials investigating cancer vaccines and oncolytic viruses in the treatment of hepatocellular carcinoma according to ClinicalTrials.gov

Product	Phase	Estimated completion	Study/identifier
OV telomelysin (OBP-301)	I	April 2021	NCT02293850
DC vaccine	II	April 2022	NCT04317248
DC vaccine + pneumococcal 13	I	May 2022	NCT03942328
OV M1-c6v1	I	October 2022	NCT04665362
DC vaccine	I	June 2023	NCT04147078
DSP-7888 PV + nivolumab or pembrolizumab	I/II	February 2024	NCT03311334
DNAJB1-PRKACA fusion kinase vaccine	I	March 2024	NCT04248569
TAEK-VAC-HerBy vaccine	I/II	December 2024	NCT04246671

DC: Dendritic cell; OV: Oncolytic virus; PV: Peptide vaccine.

cytokine analysis[126]. Although DCs seem to be an attractive treatment modality, further research is required to establish better *in vitro* handling of DCs, better methods of cellular delivery as well as further adaptation to the in-vivo environment following cellular administration.

Oncolytic virus therapy

The use of exogenously administered viruses has paved way as a new and promising modality of targeting cancer cells and promoting neoplastic destruction following replication and subsequent lysis of tumor cells. Such actions allow for the release of antigens after lysis, which triggers antitumor activity[29]. One such virus is Pexa-Vec (pexastimogene devacirepvec, JX-594), a modified pox virus expressing granulocyte-macrophage stimulating factor that activates both innate and adaptive immune responses, remaining as the leading oncolytic virus of interest when combating HCC [127,128]. A randomized phase II trial conducted in patients with advanced HCC, demonstrated dose-related significant improvement in OS at high doses (14.1 mo) compared to low-dose treatment (6.7 mo), coupled with tumor regression involving one complete response[129]. Current interest has sparked trials with combination of common therapies. We eagerly await the results of Pexa-Vec with nivolumab (NCT03071094), and Pexa-Vec with sorafenib (PHOCUS; NCT02562755) for treatment of patients with advanced HCC.

CONCLUSION

Treatment of HCC has made significant strides over the past decade since the approval of sorafenib as the first line, standard of care, molecular based treatment in 2007. More recently over the past few years four novel drugs: lenvatinib, ramucirumab, regorafenib, cabozantinib, have proven to be both safe and efficacious leading to approval for clinical use as an alternative or in addition to sorafenib. Clinical trials investigating the check point inhibitors atezolizumab, ipilimumab, nivolumab, and pembrolizumab have proven efficacy and identified new treatment modalities for HCC. As discussed in the paper adoptive immunotherapy including CAR-T cells, TCR engineered T cells, CIK cells, TIL cells, NK cells, DCs, and various vaccines have proven to be a promising therapeutic strategy for the treatment of HCC, however there are still many obstacles that need to be overcome prior to clinical application. Such challenges include identification of the ideal targeted antigen, overcoming the heterogenous and immunosuppressive environment of HCC, ensuring adequate delivery and persistence of immune cells, avoiding toxicity, and continued development of safe and cost-effective measures for cellular based treatment production and subsequent delivery to patients. The differences displayed between hepatic tumors amongst individuals across various stages of liver disease can possibly explain the difference in both clinical effects and adverse outcomes of immunotherapies studied. Therefore, we believe that the future of HCC treatment lies in the prospect of combined treatment modalities to exhibit synergistic effects, which have already entered various clinical trials as described

within this paper. While most completed and ongoing studies focus on individuals with adequate underlying hepatic function (Child-Pugh Class A), it is imperative to expand studies to other patient subgroups including those with early-stage disease and those undergoing palliative treatment. We believe that continued investigation over the coming years will overcome current barriers and lead to the development of novel and multifactorial immunotherapeutic strategies allowing for great advances in the treatment of HCC.

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MINIREVIEWS

COVID-19: Where is the treatment?

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Abstract

Even though the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is related to SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), identifying effective and safe therapeutic strategies remains challenging. In search of finding effective treatments to eradicate the virus and improve disease symptoms, scientists are exploring possible therapies such as anti-viral, anti-malaria, immune therapy, and hormone treatments. However, the efficacy of these treatments was not validated on either SARS-CoV or MERS-CoV. In this study, we have reviewed synthetic evidence achieved through systematic and meta-analysis of therapeutics specific for SARS-CoV-2 and observed that the use of the above-mentioned therapies had no clinical benefits in coronavirus disease 2019 patients and, conversely, displayed side effects.

Key Words: COVID-19; Meta-analysis; Therapeutics; Anti-viral drugs; Immune therapy; Corticosteroids

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Core Tip: The outcomes from these studies, supporting the use of anti-corona therapies, remain mostly inconclusive and uninspiring, so far, because of the lack of evidence, methodological flaws, missing data entries, risk of bias, publication bias, heterogeneity of outcomes and the number of subjects included in the respective studies. High-quality

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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data from more stringent studies involving large samples, particularly randomized clinical studies, and caution on when to employ the treatments are needed.

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INTRODUCTION

From the establishment of PRISMA guidelines in 2009, it is obvious that the importance and use of meta-analysis is growing at an unprecedented rate in scientific explorations. Meta-analysis is a statistical approach for evaluating the pooled data from various original research studies to provide quantitative, concise, and up to date knowledge[1]. Meta-analysis provides research outcomes *via* scientific synthesize through investigating the size of the effect or overall effect. This statistical analysis played a profound role in providing an evidence-based tool and in clarifying, superficially, paradoxical outcomes in several scientific domains; thus, eliminating controversy and criticism over particular study outcomes[1]. Though the term "meta-analysis" was born in the 1970s, currently the use of this tool has extended from medical sciences to other fields like physiology, conservation, evolution and ecological sciences; this infiltration strongly suggests that meta-analysis is replacing narrative reviews as an alternative, objective and instructive way of recapping biological concepts[2]. Synthesis of evidence from meta-analysis should become a common practice in order to maximize the value of scientific study in primary experimental research. Meta-analysis is very crucial to make progress in biological, medical, policy and conservation applications since these fields are greatly dependent on evidence-based outcomes[1,2]. Meta-analysis has aided in finding patterns, building projections, achieving generalizations and creating evidence-based conclusions in several research branches including oncology[3], obesity[4], pathophysiology[5], drug discovery[6] and diagnostic test accuracy[7]. Moreover, the exploration of corona virus and its therapeutic choices using meta-analysis is growing, even though the amount of original research articles over the topic are limited and these statistical outcomes are creating a corner to reach vast generalizations. Very recently, multiple meta-studies have reported their evidence-based outcomes with traditional medicines, anti-viral drugs, immune boosters, immunotherapy and use of hydroxychloroquine as treatment options in association with corona viral infection[8-12]. These meta-studies hoped to use existing evidence and provide the likability of treatment success on novel corona virus.

In December 2019, an outbreak of coronavirus disease 2019 (COVID-19) in the city of Wuhan, China, spread across the globe within a short period of time and became the latest public health emergency at the international level[13]. As of June 15, 2020, COVID-19 has been recognized in 213 countries and territories, with a total of 7805148 confirmed positive cases and with a total of 431192 fatalities. Infection control and recovery measures are necessary to prevent the current pandemic situation. It has been observed that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can bring asymptomatic, systemic or respiratory disorders in subjects infected with it. COVID-19 disease is characterized by serious upper respiratory illness including lung failure and pneumonia[14], where the cause of disease was COVID-19 virus [World Health Organization (WHO) named on February 11, 2020] and has been identified as a new novel coronavirus, which is now confirmed as SARS-CoV-2[13,14]. Earlier two major outbreaks of corona viruses occurred, namely Middle East respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV, posing a great threat to public health; however, these diseases were not deemed as pandemics. The SARS-CoV-2, which emerged in 2002, is a zoonotic corona virus similar to that of SARS-CoV[14,15]. As COVID-19 has triggered enormous human casualties and serious economic losses globally, an understanding of the ongoing situation and the development of strategies to contain the virus's spread are urgently needed. COVID-19 has caused a disturbed lifestyle, colossal human deaths, and pressing industrial losses globally and within a short period. This outbreak calls for urgent and effective measurements, anti-viral

therapeutics, and the establishment of effective strategies to restrain the virus. Several scientific explorations, particularly meta-studies are providing a great amount of evidence to adapt various therapeutic choices including immune therapy, anti-viral drugs and even the use of traditional medicines to treat COVID-19 infection. Hence, in the current review, we aim to review the status of all meta-studies published from 2019-2020 focusing on therapeutic options for COVID-19 to highlight future directions in the development of safe and successful therapeutic agents to prevent the viral disease (Figure 1).

THE PATHOGENESIS OF COVID-19

Though the initial transmission of COVID-19 was reported in Wuhan City, China, the actual source, reservoirs or intermediate carriers of the virus are still unknown. The latest COVID-19 viral genome has 88% similarity with SARS-CoV, which are derived from bats. The similarity also suggests that no birds or reptiles can host this virus except mammals[16]. Though the information on primary reservoirs of COVID-19 remain unclear, the transmission from person-to person *via* virus laden released during sneezing, coughing, or direct contact with infected person was reported[17]. Studies also confirmed that there is no transmission of virus from mother to child during pregnancy[18]. The first step of viral infection is the binding of viral spikes with cell-surface receptors of host cells and subsequent fusion with the plasma membrane, specifically on the epithelial cells of the lungs. Studies have investigated and confirmed that COVID-19 shares similarity in the receptor-binding domain of the SARS-CoV and interacts with angiotensin-converting enzyme 2 receptor (ACE2) present on the upper respiratory tract cells to gain invasion into the host system[17]. Moreover, a very recent study identified a group of human's proteins able to interact with SARS-CoV-2 proteins and these host proteins exhibit a range of functions at the cellular level (including DNA replication, vesicle traffic, mitochondrial, nuclear transport, cytoskeleton, lipid modifications, epigenetic regulators and ubiquitin ligases)[19].

It has been observed that SARS-CoV-2 can bring asymptomatic, systemic or respiratory disorders in subjects infected with it[20,21]. An incubation period of 5.8 d was seen in patients infected with COVID-19 with no symptoms and only after this incubation phase, the symptoms of COVID-19 appear from mild to life-threatening illness within 6 to 41 d, with an average of 14 d[20,21]. The appearance of symptoms after the incubation period depends on age as well as the individual's immune system. The common systemic symptoms of COVID-19 infection are fever, dry cough, fatigue, headache, dyspnoea, gastrointestinal symptoms, lymphopenia and haemoptysis[22]. The life-threating respiratory disorders such as pneumonia, respiratory distress syndrome, acute cardiac injury, serum SARS-CoV-2 viral load (RNAaemia), and prevalence grand-glass opacities in lungs were also reported[22]. Patients infected with COVID-19, who experienced breathing difficulties and pneumonia, also had high levels of pro-inflammatory cytokines and chemokines (granule cell stimulating factor, granulocyte-macrophage colony-stimulating factor, interleukin (IL)1-β, IL1RA, IL7/8/9/10, fibroblast growth factor 2, interferon γ, IP10, MCP1, MIP1α/β, platelet-derived growth factor B, tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor A) in the serum; these cytokines and chemokines are related in promoting disease progression[23]. At present, the world is not prepared to face pandemics like COVID-19 and is suffering from its consequences. Until today, there are no functional therapeutic drugs to treat the COVID-19 viral infection. However, COVID-19 patients are receiving supportive care, oxygen supply *via* ventilators and fluid management to overcome symptoms; nonetheless, there are tremendous efforts for vaccine development are undergoing. The global research community and pharma industries are working closely to find a cure for COVID-19 yet remain unsuccessful due to lack of existing evidence about druggable agents, which can provide a safe and sustainable cure for COVID-19 infection. The one possible way to accelerate the discovery process is to look for relatable drug agents that are primarily used in pneumonia, SARS-CoV and MERS-CoV infections. A wide variety of druggable agents or strategies those are known to work against deadly viruses such human immunodeficiency virus and Ebola can also be adapted; however, a clinical validation is needed. To accelerate the discovery of a druggable agent, a clear validation and generalizations of existing information on corona viruses and their killer is useful. To achieve such goals meta-studies are the only source. Hence, in the following sections, we will highlight the evaluations of meta-studies conducted on various possible drug agents

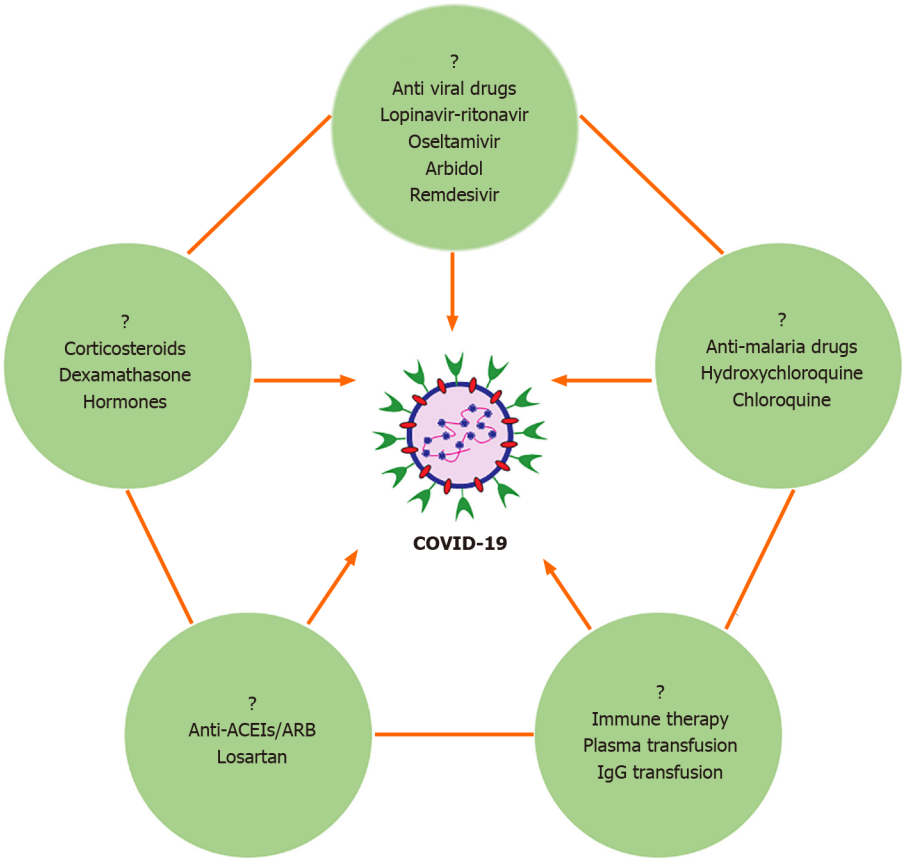


Figure 1 Coronavirus disease 2019 and its possible treatments. COVID-19: Coronavirus disease 2019; ACEI: Angiotensin-converting enzyme inhibitors; ARB: Angiotensin receptor blockers; IgG: Immunoglobulin G.

against COVID-19 to find the most suitable and reliable cure.

Meta-analysis and COVID-19 therapeutics

The synthesis of scientific results from pooled data can help us to compare existing results in order to comprehend epidemiology, mortality, management choices, risk assessment and efficiency of prophylactic strategies against COVID-19. When we set the aim to explore the meta-studies on COVID-19 treatment efficiencies, we were unable to find such studies in the databases. The reason could be due to the lack of sufficient or compelling experimental evidences or clinical investigations to perform meta-analysis on prophylactic strategies against COVID-19. However, a few groups tried to find the pattern in aetiology, comorbidities and pathological variations and risk factors of COVID-19 at length[24-27]. Zhong *et al*[28] tried to perform systematic cum meta-analysis on the efficiency and safety of prophylactic strategies against COVID-19. Since the available data on such a topic was less, they tried to evaluate SARS-CoV and MERS-CoV therapies to find relatable and promising treatment options for SARS-CoV-2 infection[28]. In their meta study, antiviral drugs such as lopinavir/ritonavir, ribavirin and anti-malaria drug hydroxychloroquine based clinical data on SARS-CoV, MERS-CoV and COVID-19 was evaluated and found that, altogether, there was an improved mortality rate and reduced clinical development and radiographical improvement but no clear conclusion on the eradication of virus, the incubation phase, the prevalence of acute respiratory disease syndrome and adverse events[28]. However, a subgroup evaluation confirmed that use of ribavirin and corticosteroids in combination had a positive effect on reducing mortality and hydroxychloroquine, which was demonstrated on radiographical outcomes alone. In addition, use of a combination of lopinavir/ritonavir exhibited better eradication of the virus and improved radiographical appearances with a low prevalence of acute respiratory disease syndrome. Keeping the side effects of the drug combination tested into consideration, the quality of verification on most end results were very low and disappointing[28]. Though the meta-analysis failed to draw direct conclusions, due to the heterogeneity and low quality of evidence and indications, the study is still useful for clinicians to thoroughly acknowledge the dos and don'ts of individual anti-

coronavirus agents on efficacy and safety. On the other hand, Etoom *et al*[29] recently commented on one meta study published by Hu *et al*[30] titled "Prevalence and severity of COVID-19: A systematic review and meta-analysis". The former group thinks a much deeper and careful statistical strategy is needed to evaluate the available evidences on COVID-19. They also suggested that meta-analysis data should be made available, especially in the case of COVID-19, since the number of research explorations are growing immensely and performing new meta-analysis would be easier and quicker[29,30]. We believe, at this stage meta-studies on COVID-19 and therapeutics efficiency might take time because of the lack of sufficient empirical data and since the rate of research investigations are actually yielding low successful outcomes. Nonetheless, we look forward to see an increase in meta-studies aimed at COVID-19 research outcomes. It is noteworthy to mention that, though there were no meta-studies on efficiency of prophylactic strategies against COVID-19, there are a few systematic reviews on the same subject. Meta-analysis and systematic reviews stand atop in estimating the quality of evidence known as "evidence pyramid". Systematic review does not require statistical analysis, but provides a comprehensive synopsis of scientific literature to a specific research question.

Systematic reviews and COVID-19 therapeutics

In order to curtail the current SARS-CoV-2 global crisis, rapid diagnostics and effective therapeutics are the key potential interventions, which are currently occurring. Moreover, the lessons from previous outbreaks have shown that earlier therapeutics can still be questionable for the use in the current pandemic. Among the ongoing clinical investigations, some of them are testing against SARS-CoV and MERS-CoV while the rest are focused on SARS-CoV-2; however, currently, there is no success of effective therapeutics specific to SARS-CoV-2. The ways to eradicate SARS-CoV-2 include anti-viral drugs, immune therapy, immune boosters, vaccines, anti-malarial drugs, monoclonal antibodies and convalescent plasma, which are majorly monitored by pharma and research investigators. The ultimate goal is to develop anti-corona therapeutics; to accelerate such processes, every effort made is accountable and systematic generalization of such progresses play an important role in deciding the efficacy and safety of individual anti-coronavirus agents. In this hour of need what we want is a magic bullet to stop COVID-19; however, it is not easy to identify, testify, validate and get approval for such magic bullet. Constant efforts from biologists, pharmacists and policy makers are needed to evaluate the efficacy of anti-corona therapies and we are running out of time. We have highlighted the evidence collected through systematic analysis on anti-corona therapies to bring a comprehensive, evidence-based evaluation under one roof to further increase the understanding on the current success rate of anti-corona therapies. An overview of some of the most relevant systematic reviews (SR) and meta-analysis (MA) studies conducted on therapeutic strategies specific to COVID-19 are given in Table 1.

Systematic evaluation of anti-viral drugs specific to SARS-CoV-2

The ongoing antiviral drugs against COVID-19 are mostly similar to MERS-CoV and SARS-CoV-1 studies. A recent study conducted a systematic review on the current clinical settings of antiviral therapies against COVID-19[8]. The study also conducted a similar evaluation on MERS-CoV and SARS-CoV-1 studies to filter potential antiviral drugs. In their analysis, only one clinical investigation involving lopinavir-ritonavir in management of COVID-19 was found, where the treatment had no benefit in 199 severe COVID-19 patients[8]. It is noteworthy to mention that other observational studies, where anti-viral drugs such oseltamivir, lopinavir-ritonavir, lianhuaqingwen capsule, arbidol and interferon were used in the management of COVID-19, could not yield positive conclusions due to the lack of data recording and appropriate sample sizes[8]. Most interestingly, the team could not find any clinical settings where the effects of anti-viral drugs were tested against MERS-CoV and SARS-CoV-1 infections [8]. From the above conclusions, we believe that the current pandemic is more challenging to curb and will be difficult and even more challenging to develop an ideal anti-viral drug to manage severe COVID-19 diseases. Another team also aimed to evaluate the prophylaxis of anti-retroviral drugs on COVID-19 using systematic review to generate strong evidence to support anti-viral drugs as the first line of treatment. They reported the availability of 21 observational studies and two randomized trials, which showed the end results on the use of lopinavir-ritonavir on MERS-CoV, SARS-CoV-1, and COVID-19 patients[31]. From their evaluation, it was suggested that there were no clinical benefits from randomized trials, no inconclusive outcomes from observational studies and a low body of evidence across all major end points, indicating that the use of lopinavir-ritonavir anti-viral drugs as the first line of

Table 1 Synthetic of evidence on coronavirus disease 2019 specific therapeutic strategies

Nature of therapeutics	PMID	Study type	Therapeutic	Benefits	Conclusion
Anti-viral drugs	32360583	SR	Lopinavir-ritonavir or ribavirin	Improved mortality rate, radiographical improvement and reduced clinical development	Inconclusive evidences, low quality of evidence and heterogeneity of interventions
	32309809		Lopinavir-ritonavir or Arbidol or Oseltamivir or Lianhuaqingwen capsule or interferon	No benefits in 199 subjects	Side effects. Inconclusive evidence lacks of data recording. Sample size
	32493740	SR & MA			
	32293807	SR	Lopinavir-ritonavir	No clinical benefits	Adverse side effects. Inconclusive outcomes. Low body of evidence. Small sample size
	32506110		Remdesivir	No clinical benefits	Inconclusive outcomes high-quality evidence well-designed studies. Safety
	32378648				
Immune therapy	32406927	SR	Plasma transfusion	Had beneficial outcomes	Side effects. Inconclusive outcomes. Very low-certainty. High risk of bias. Low reporting quality
	32272396		Plasma therapy or hyperimmune immunoglobulin transfusion	Had beneficial outcomes	More evidence. Promising strategy. Sample size. Lack of control group
	32527348				
Anti-malaria drugs	32359203	SR	Hydroxychloroquine/Chloroquine	Had beneficial outcomes	Inconclusive outcomes. Methodological flaws. Risk of bias. Lack of evidence
	32281213			No clinical benefits	Lack of evidence. Safety issues
	32468425				Methodological flaws. Small sample size. Safety issues
	32173110				Lack of evidence. Safety issues. Methodological flaws. Small sample size
	32519281				
Hormone therapy	32283144	SR & MA	Corticosteroids	No clinical benefits	Lack of evidence. Adverse side effects. Methodological flaws. Caution needed
	32409522	SR			
	32372026				
	32391369				
Anti-hypertension drugs	32542337	SR & MA	ACEI/ARB	Had beneficial outcomes	Conflicting results, scarce existing data. Diverse study types. Inconsistent clinical studies, more RCT needed

ACEI: Angiotensin-converting enzyme inhibitors; ARB: Angiotensin-receptor blockers; RCT: Randomised controlled trial; SR: Systematic reviews; MA: Meta-analysis.

treatment is not efficient on COVID-19 patients[31]. The team highlighted that reliability of the proof of end results across MERS-CoV, SARS-CoV-1, and COVID-19 is very low. They also suggested that, in addition to small sample size, the dose, duration and timing of the treatment was not uniform. Moreover, a combination of antiviral drugs along with other interventions may have given rise to the disclosed outcomes [31]. Though both studies differed on trivial aspects of anti-viral drug efficacy against COVID-19, MERS-CoV and SARS-CoV-1 infection, the studies did agree on one common finding, that the use of lopinavir-ritonavir on severe COVID-19 is not efficient. Currently, nearly twenty-five clinical trials are registered and each of the plans are investigating the efficacy and safety of anti-viral agents, including cobicistat, ritonavir, darunavir, lopinavir-ritonavir and tenofovir alafenamide fumarate.

Systematic evaluation of immune therapy specific to SARS-CoV-2

The reason behind choosing immune therapy against COVID-19 is because of the presence of clinical features, such as lymphopenia, increased inflammatory cytokines,

chemokines and reduced IFN-γ expression in T cells, which indicate suppression of the host immune system against sever COVID-19 infections[32]. The strategies to boost the host immune system through neutralizing antibodies or vaccines are focused on provoking the immune system to fight against COVID-19. However, in the case of COVID-19, the most popular known immune therapy is hyperimmune immunoglobulin transfusion or plasma therapy with nearly 48 studies aimed to evaluate hyperimmune immunoglobulin transfusion or plasma therapy for COVID-19 infected people. A rapid systematic review conducted by Valk *et al*[33] assessed the risks and benefits of using plasma transfusion as a potential immune therapy for COVID-19[33]. They reported that the majority of studies identified adverse side effects (grade 3/4) and that the quality of the reported data was low on plasma transfusion. The data deposited on plasma transfusion studies was highly inconsistent, making it difficult to draw outcomes with certainty[33]. Moreover, some of the controlled non-randomised or randomised controlled investigations are still occurring and have not reported any data regarding the harms and benefits of convalescent plasma therapy[33]. Though the plasma therapy looks promising, the current, global systematic analysis it is not efficient and safe. However, the effectiveness and safety of plasma transfusion remain elusive when compared with other immune therapeutic strategies.

Another study performed a systematic review on probable immune therapies for COVID-19. In this study, the authors included evidences from MERS-CoV, SARS-CoV and SARS-CoV-2 infections, in which the safety and efficacy of immunotherapy was investigated[34]. The highlights of the study primarily come from a single ethnic group (in China); evidence was reported in clinical settings, where the use of plasma therapy (300 subjects), hyperimmune immunoglobulin transfusion (80 subjects), thymosin in combination with camrelizumab (120 subjects) and tocilizumab (188 subjects) against COVID-19 were tested[35]. Though the outcomes of the studies with plasma therapy or hyperimmune immunoglobulin transfusion reported clinical improvements in COVID-19 patients, the evidence to support such therapies fails to provide certainty and demands for further investigations on a large-scale and more diverse population[34]. Moreover, the end outcomes of thymosin/camrelizumab combination therapy and tocilizumab against COVID-19 have yet to be tested. However, the study recommends to test the efficacy and safety of interventions like viral-vectors, vaccines, nanoparticles, and monoclonal antibody against COVID-19 infection, since those have been tested for SARS-CoV in non-clinical settings[34]. Another study performed a systematic analysis to determine the efficacy and safety of immune suppressive/stimulating drugs, such as non-steroidal anti-inflammatory drugs, TNF-α inhibitors, IL-6 inhibitors and Janus kinase/signal transducers and activators of transcription pathway inhibitors. These studies reported that no definite supporting evidence is available from clinical investigations; hence, they recommend clinical investigations using such drugs as promising immune therapeutics, since their efficacy was proved in *in vitro* studies[12]. Whatever approach was adapted by the above-discussed systematics, the end outcome, in terms of using immune therapy for COVID-19, remains dark. The literature is filled with reviews discussing the many possible approaches to prevent COVID-19, but what we need at this moment is more evidence-based studies than narrative opinions.

Systematic evaluation of anti-malaria drugs specific to SARS-CoV-2

In an unusual way, the use of anti-malarial drugs to cure viral infections has become quite popular lately. Malaria is caused by a parasite, *Plasmodium,* and COVID-19 disease is caused by the human corona virus; there are no structural or pathological similarities between the virus and the parasite. However, they both definitely increase body temperature upon infection. The earlier use of anti-malaria drug, such as chloroquine, against human corona viral infections in mouse models was reported in 2009[36]. Due to the public health emergency from SARS-CoV-2, every possible means to prevent viral infections was predicted and being tested. Hence, anti-malaria drugs are currently one of the drugs being evaluated for their efficacy and safety against SARS-CoV-2 in humans. A systematic review conducted found evidence regarding the effectiveness of anti-malaria drugs as anti-viral drugs to prevent SARS-CoV-2[37]. The team reported seven clinical trials, which were evaluating hydroxychloroquine/chloroquine as therapy for SARS-CoV-2, as complete. The end outcomes of the study suggest that both hydroxychloroquine/chloroquine were effective compared to supportive care or anti-viral drug treatment of SARS-CoV-2[37]. However, the outcomes are not reliable, since the evaluated studies has methodological flaws and risk for bias, indicating a lack of evidence to support anti-malaria drugs on SARS-CoV-2. However, hopefully, the data from ongoing trials may provide some evidence in the future[37]. Another study found similar outcomes upon systematic revision of prophy-

lactic outcomes of hydroxychloroquine and chloroquine against SARS-CoV-2; here, the team concluded that there is a lack of evidence to support hydroxychloroquine and chloroquine routine use and there are potential safety issues, which need to be further evaluated[38]. Another study screened nearly 663 articles and 12 clinical trials, validated the use of hydroxychloroquine and chloroquine against SARS-CoV-2 and found that some of the studies had better clinical outcomes with hydroxychloroquine or combination of azithromycin/hydroxychloroquine use in COVID-19 patients. However, these studies also had major flaws in their methodology. Moreover, a few studies showed adverse and opposite outcomes with hydroxychloroquine[39]. Similarly, another study reported similar observations and came up with a few recommendations, like employing a proper approach based on Monitored Emergency Use of Unregistered Interventions or WHO guidelines in upcoming clinical settings, especially with the use of anti-malaria drugs[40]. We also believe that a better-quality and stringent studies design and inter-relatable data from clinical trials originating across the globe are needed to clear the air regarding the use of hydroxychloroquine and chloroquine against SARS-CoV-2. It is noteworthy to mention that not only systematic reviews but also meta-analyses confirm the ineffective role of hydroxychloroquine in treating COVID-19 patients[9]. The Meta study confirms that there was no effect on viral eradication and a significant mortality rate was seen in COVID-19 patients treated with hydroxychloroquine[9].

Systematic evaluation of corticosteroids specific to SARS-CoV-2

Steroid hormones exhibit an inhibitory role on inflammation, when used in viral pneumonia; hence, many physicians recommended corticosteroid therapy as a possible treatment for patients with COVID-19. Though corticosteroid does not affect the virus directly, this therapy may help in managing severe inflammation and regulate homeostasis. Thousands of people infected with COVID-19 were treated with corticosteroid alone or in combination with anti-viral drugs. Meta-analysis and systematic reviews conducted on finding evidence in support of corticosteroid therapy for SARS-CoV-2 suggested that hormone therapy is ineffective and provokes adverse side effects.

A study by Yang *et al*[41] found that only patients with severe COVID-19 require hormone therapy and routine use of corticosteroid; these patients showed an increased mortality rate, bacterial infection and low blood potassium levels[41]. A caution must be taken while considering corticosteroid as a therapeutic option for mild symptoms [42]. Overall, the study finds inconclusive results, such as sample size, risk of bias in outcomes and the lack of data from multi-centre clinical trials[41]. A different meta-analysis ruled out the safety and efficacy of corticosteroids in SARS-CoV-2 infections. When they tested the virus' clearing effect by corticosteroids, they observed a slow virus clearing rate in two studies[43]. The meta-analysis concludes that there was no improvements in the death rate or the length of stay, which was accompanied by adverse effects[43]. Due to predominance of observational trials in the meta-study, a demand for confirmation from randomized trials to overcome the publication bias is needed[43]. Additional studies also concluded that the current evidence does not fully recommend the use of corticosteroids in SARS-CoV-2 infections; however, a few outcomes recommend the use of methylprednisolone to decrease the mortality rate in severe SARS-CoV-2[44,45].

Other notable mentions

While we discussed the most popular strategies employed in treating COVID-19, it is also important to explore other strategies employed in curing SARS-CoV-2 infection, such as remdesivir, anti-hypertension drugs and Traditional Chinese Medicine. Remdesivir is a broad-spectrum, anti-viral nucleotide analogue that has gained significant attention lately. In preclinical studies, remdesivir has been known to block a range of corona viruses and improve lung function therapeutically; however, the efficacy of remdesivir in COVID-19 patients remains short and scattered. The drug remdesivir received approval to be used under "Emergency Use Authorization" against severe COVID-19 cases, but is still awaiting approval by Food and Drug Administration. A recent systematic review conducted assessed the current evidence on the efficacy and safety of remdesivir and found favorable evidence as a first line treatment option in SARS-CoV-2[46]. The study reported that in order to confirm and recommend remdesivir as high quality and bias free, evidence from clinical settings is needed. Moreover, clinical settings should qualify with larger sample sizes, constrictive design and well-recorded data to synthesize an effective con-clusion[46, 47]. Furthermore, the future is hopeful with these ongoing trials and these studies may provide effective evidence on the benefits of remdesivir in COVID-19[47]. The second

choice that was opted to treat SARS-CoV-2 infection was inhibitors of the angiotensin receptor or ACE, a possible way to block viral interaction with receptors on lung epithelia cells. Meta studies on Angiotensin-converting enzyme inhibitors/angiotensin-receptor blockers (ACEIs/ARBs) inhibitor-based treatment on SARS-CoV-2 concluded that using inhibitors of ACEIs/ARBs can be continued but large studies such as randomised controlled trial are needed and additional evaluation on the relationship between polymorphism of ACE2 and its inhibitor is a must in the future investigations[48,49]. On the bright side, the upcoming outcomes from clinical trials (NCT04312009 and NCT04311177) using anti-ARB drug (losartan) in COVID-19 hold promising insights. The third choice that was opted to treat SARS-CoV-2 infection was a combination of Western medicine with TCM. One study aimed to investigate the benefits and harms of herbal medicine and Western medicine over COVID-19[50]. The amalgamated therapy rapidly increased the overall effective rate with better clinical outcomes in COVID-19 patients with zero side effects. However, additional evidence from randomized clinical trials may help to validate the benefits or harms of integrated medicine in the treatment of COVID-19[50]. Another study also showed that integrated medicine has beneficial effects when compared to Western medicine alone. The combination therapy did not yield any adverse effects in COVID-19 subjects. The number of studies included quality of data but poor methodologies were adapted in tested studies; a demand for more evidence with good quality to make definite decisions about combination therapy is needed in the future[11]. Effective vaccines are curial in the long-term to prevent rapid transmission of SARS-CoV-2 infections but developing vaccines is time consuming. However, the current crisis is pushing the limits of vaccine development and a few vaccines are being investigated in clinical trials right now. Table 2 provides the details of vaccines developed and their current clinical state specific to SARS-CoV-2. The preceding vaccines belong to DNA, RNA, inactivated viruses, recombinant viral spike proteins, dendritic cells, minigenes and viral vector-based systems. A vaccine developed by Oxford University and AstraZeneca is the most progressive one to enter phase III study settings; this vaccine serves as hope for a promising cure for SARS-CoV-2. Clinical investigations, clinical clearance and approval from the governing body are prerequisites for any drug, vaccines or therapeutic strategies created. Currently, apart from the ones discussed earlier, a wide range of anti-viral drugs, vaccines, cell therapies and anti-bacterial therapeutics are ongoing to determine the safety and efficacy specific to SARS-CoV-2.

SARS-CoV-2 specific drug targets under clinical investigation

Even though the success rate was low and search for suitable, relatable therapeutic drugs against SARS-CoV-2 continues with extensive hard work. In this section we focus on some of the possible upcoming therapeutics which are under early as well late stages of clinical monitoring. A pilot study pursing the benefits of using amniotic fluid (NCT04319731) or mesenchymal stromal cells from card tissues (NCT04399889, NCT04345601 and NCT04276987) for patients with COVID-19 and use of amniotic fluid was an approved strategies to minimize inflammation, tissues damage in humans. Use of rhDNase1 inhalation to trap neutrophils increased in circulation due to elevated inflammation is under evaluation against SARS-CoV-2 infection (NCT04409925). Anti-IL-6 antibody named Sarilumab has entered clinical evaluation as a potential mediator to interrupt cytokine-linked respiratory injury caused by SARS-CoV-2 infection (NCT04386239). Mouth or nose cleaning and gargling with povidone-iodine solution is currently undergoing clinical examination (NCT04393792), since povidone-iodine killed SARS-CoV-2 virus effectively in *in vitro* studies[51]. A phase III study aimed to rule out dosing with Sildenafil tablets in SARS-CoV-2, since it was approved by WHO for the prevention of pulmonary arterial hypertension (NCT04304313). A Tyrosine kinase inhibitor, Imatinib mesylate blocked inflammatory responses in invitro, *in vivo* and in few clinical trials, is under further examination in a phase III trial on SARS-CoV-2 and hoping to observe reduction in disease severity and inflammation (NCT04422678). Baricitinib, anti-Janus kinase inhibitor was approved to treat rheumatoid arthritis earlier and preclinical studies confirm that it can lower or prevent entry of viruses in to epithelia cells and reduce cytokine release, is under phase III clinical monitoring to use on SARS-CoV-2 (NCT04320277). The use of repurposed bacterial mucosal vaccines Bactek-MV130 in the form of spray is undergoing phase III trial to provide benefits for COVID-19 induced mild pneumonia (NCT04363814). Based on preclinical evidences where increased circulatory Vascular Endothelial Growth Factor was seen in COVID-19 subjects, anti-vascular endothelial growth factor drug namely Bevacizumab (FAD approved to treat certain cancers), is being tested in critical or severe patients with COVID-19 pneumonia (NCT04275414). Ifenprodil, a drug used to inactivate activated neutrophils and T-cells is under phase

Table 2 Vaccines specific to severe acute respiratory syndrome coronavirus 2 under clinical investigation and their development status

Vaccine name	Vaccine type	Status	Registration ID	Date of registration	Developer
2019-nCOV	Adenovirus vaccine	Phase II	ChiCTR2000031781	10-Apr-20	Academy of Military Medical Sciences
Ad5-nCoV	Adenovirus vaccine	Phase II	NCT04341389	10-Apr-20	CanSino Biologics
AV-COVID-19	Autologous dendritic cells	Phase IB/II	NCT04386252	13-May-20	Aivita Biomedical, Inc
BBIBP-CorV	Inactivated virus	Phase I/II	ChiCTR2000032459	29-Apr-20	Beijing Institute of Biological Products & Sinopharm
BNT162	mRNA vaccine	Phase I/II	NCT04380701	8-May-20	BioNTech and Pfizer
ChAdOx1	Adenovirus vaccine	Phase II/III	NCT04400838	26-May-20	University of Oxford
Covax-19™	SARS-CoV-2 spike protein	Phase I	NCT04428073	11-Jun-20	GeneCure Biotechnologies
COVID-19/aAPC	Antigen presenting cells	Phase I	NCT04299724	9-Mar-20	Shenzhen Geno-Immune Medical Institute
INO-4800	DNA vaccine	Phase I	NCT04336410	7-Apr-20	Inovio Pharmaceuticals
LV-SMENP-DC	Lentiviral vector system	Phase I/II	NCT04276896	19-Feb-20	Shenzhen Geno-Immune Medical Institute
mRNA-1273	mRNA vaccine	Phase II	NCT04405076	28-May-20	ModernaTX, Inc
NVX-CoV2373	Recombinant Spike Protein	Phase I/II	NCT04368988	30-Apr-20	Novavax
PiCoVacc	Inactivated virus + adjuvant	Phase I/II	NCT04352608	20-Apr-20	Sinovac
V- SARS	Heat-inactivated plasma	Phase I/II	NCT04380532	8-May-20	Immunitor LLC
Vero cells	Inactivated virus	Phase I/II	ChiCTR2000031809	11-Apr-20	Wuhan Institute of Biological Products & Sinopharm

LLC: Lewis lung carcinoma; COVID-19: Coronavirus disease 2019; SARS: Severe acute respiratory syndrome.

IIb/III trial to discover the safety and efficacy in the treatment of SARS-CoV-2 infection (NCT04382924). There is no recorded evidence from all the trials motioned above, so we do not know whether anti-inflammatory drugs, rheumatoid arthritis and anti-septic solutions have any benefits in COVID-19 patients. Noteworthy to mention that the above detailed investigations are based on the evidence gathered from preclinical studies and the benefits of using these repurposed medications against COVID-19 need evidence from these study outcomes. Moreover, the safety and efficacy of these drugs on COVID-19 are forthcoming and we hope the outcomes from these studies provide conclusive, bias free evidences. It seems the new players under investigation are mainly to treat SARS-CoV-2 symptoms and have zero effect on viral load, except for povidone-iodine, which was known to eradicate SARS-CoV-2. The world is eagerly dependent on scientific and pharma community for the discovery of magic bullet to treat COVID-19.

SARS-CoV-2 therapeutic challenges and anticipations

In spite of the worldwide distress, no drug or vaccines is available under approval to treat SARS-CoV-2 infection. So far, clinical reports and synthetic evidence show that reusing of existing anti-viral, anti-malaria, immune stimulators and corticosteroids on COVID-19 disease has been unsatisfactory and unsafe. In order to escalate the identification and validation of anti-SARS-CoV-2 medicine, use of computer based high through put data analysis in finding suitable drug targets is recommended. A broad range of anti-viral agents in preclinical and clinical settings must be evaluated for their safety and efficacy against SARS-CoV-2. We believe that use of repurposed medicines for COVID-19 are going to be part of short-term strategy. In spite of the fact that these relatable treatment options have been prioritized to treat SARS-CoV-2 and the outcomes are biased. These uncovering's highlight an immediate call for novel anti-corona medicines specific to SARS-CoV-2 virus. In the current urgency there are several challenges and overcoming those challenges is crucial in developing novel anti-SARS-CoV-2 medications. One such hurdle is collection of evidence from

preclinical experiments, which is expensive and time-dependent. In the absence of preclinical data, computer-based analysis of target profiling allows either to validate or invalidate the use of predicted drug; permits to accelerate invention of novel and beneficial therapeutics against SARS-CoV-2. Though the operating cost is reduced, the safety profile of those computer-generated targets must be validated at least in clinical settings. Another limitation faced by scientist is the availability of suitable study models (cells, mice and primates) to investigate the virus-host interaction and evaluate the potency of anti-SARS-CoV-2 drugs. In addition, BSL3 Laboratories with suitable study models are very few and conducting experiments in such environments is technically difficult. The development of host-based and/or SARS-CoV-2 based clinical inventions must be prioritized since only one or two of drugs will pass through clinical settings. Toxicity, dosage, availability of drug delivery routes and some other limitations make these drugs to pass through the clinical stage. Normally, the development and clinical approval of vaccine needs more than 10 years of time and efforts, however, vaccine development programs against SARS-CoV-2 are breaking the convectional norms and working hard to launch safe and effective SARS-CoV-2 vaccines as early as possible. It can be more challenging to treat COVID-19 diseases, if the virus develops genetic mutations and/or drug resistance during treatment. Studies have reported presence of 93 possible mutations and among them majority of mutations were missense mutations and the genome of SARS-CoV-2 was found to be highly conserved[52]. Also, patients with underlying health issues such as diabetes, cancer, renal failure and women with pregnancy need exceptional care. In addition, management of COVID-19 patients has become challenging due to lack of sufficient medical staff, availability of drugs, subject recognition's, isolation and implication of control procedures and delivery of personalized care towards COVID-19 patients. New Zealand and few Asian countries managed to contain COVID-19 transmission effectively while compared with most of developed countries like United States and Europe. As the epidemic advances, hunger amid poor countries, influence of lock down on mental status of children and adults may increases. To avoid upcoming foreseeable future, systematic and unified approach is vital for managing COVID-19 epidemic. A single drug or a combination approach to preventing COVID-19 disease is needed to prevent mortality, restore the normal lifestyle and economic growth world-wide. The ideal drug must be able to kill virus load with zero or less toxicity in humans, affordable with minimum production time.

CONCLUSION

In this real-time crisis, a need for high-quality, bias-free, and effective evidence in eradicating the SARS-CoV-2 virus and reducing disease-associated symptoms with zero side effects is needed. Currently, the journal database is filled with narrative research and speculations with hypothetical reasoning; data on evidence-based research is limited. A shift in evidence originating from original research investigations and clinical studies with high-quality evidence is needed and this evidence may aid in progressing towards the development of effective and safe therapeutic options for SARS-CoV-2. Moreover, the systematic reviews and meta-studies are providing substantial evidence and clearing the air of bias for the public, physicians, and scientists. Drugs like hydroxychloroquine/chloroquine or remdesivir have gained a lot of attention in the public and the media; these drugs have also gained support from experts and are now being characterized as effective therapeutic drugs for SARS-CoV-2 but scientifically it is unwise to recommend these drugs to treat SARS-CoV-2 without caution. Currently, there are no effective anti-viral agents, immune therapy, or vaccines against SARS-CoV-2 but the future is filled with only hope. The current scientific evidence hold inconclusive outcomes because of the low number of studies conducted, low sample size, flawed study designs, publication bias, heterogeneity, missing data records, quality of evidence, ethnicity and presence of adverse effects. However, these flaws cannot stop current and future investigations to identify, characterize and validate possible therapeutic innovations specific to SARS-CoV-2.

Future directions

So far, considering the trajectory of COVID-19 pandemic, the primary need to control the transmission of the disease is to practice physical distancing, wearing masks, keeping hands off from surfaces, and washing them with proper detergent. These rules have somewhat provided the needed protection. The public is well aware of the facts to restrain themselves from being exposed. Developing surfaces or masks that

can kill the virus within seconds once contact made could be a way to prevent highly transmissive virus-laden droplets that are just released. Coming to the scientific role in finding a cure, it is very crucial to understand the genetic profile of the virus at the molecular level and use this knowledge to build safe tools to screen, identify and develop therapeutics for the prevention and treatment of SARS-CoV-2. Currently, no laboratory in the world has whole virus to study in detail. Hence, it is only fitting to build the tools and resources that can be utilized for evaluating therapeutic efficacy. The journey to finding a vaccine or an anti-viral therapy specific to SARS-CoV-2 must start from the basic research integrated with advanced technologies such as artificial intelligence, computational biology, nanotechnology, and genome-wide association studies. The efforts made in the past to find the cure for COVID-19 though failed but provided immense knowledge on what not to practice and open new doors of possibility. Currently, applying a single therapeutic strategy, or even a combination of strategies is not enough to cure diseases including cancers and infectious diseases. In the end, prevention is better than a cure.

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MINIREVIEWS

Immune response evaluation criteria in solid tumors for assessment of atypical responses after immunotherapy

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Abstract

In 2017, immune response evaluation criteria in solid tumors (iRECIST) were introduced to validate radiologic and clinical interpretations and to better analyze tumor's response to immunotherapy, considering the different time of following and response, between this new therapy compared to the standard one. However, even if the iRECIST are worldwide accepted, to date, different aspects should be better underlined and well reported, especially in clinical practice. Clinical experience has demonstrated that in a non-negligible percentage of patients, it is challenging to determine the correct category of response (stable disease, progression disease, partial or complete response), and consequently, to define which is the best management for those patients. Approaching radiological response in patients who underwent immunotherapy, a new uncommon kind of target lesions behavior was found. This phenomenon is mainly due to the different mechanisms of action of immunotherapeutic drug. Therefore, new groups of response have been described in clinical practice, defined as "atypical responses," and categorized into three new groups: pseudoprogression, hyperprogression, and dissociated response. This review summarizes and reports these patterns, helping clinicians and radiologists get used to atypical responses, in order to identify patients that respond best to treatment.

Key Words: Response evaluation criteria in solid tumors; Tumor response; Pseudoprogression; Hyperprogression; Dissociated response

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Core Tip: Atypical responses are frequent events in the immunotherapy era. On these bases, it is fundamental to summarize and recap the most common and important response manifestations to help clinicians in everyday practice. Here, we present the three most common clinical and radiological patterns of response to immunotherapy: pseudoprogression, hyperprogression, and dissociated response, reporting important studies to identify the different behavior and guarantee the best management, strengthening the communication skills between specialists.

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INTRODUCTION

In the last few years, new therapies such as immunotherapy have been experimented with and introduced into clinical practice for the treatment of oncologic patients. Immunotherapy is a type of treatment that involves the immune system to fight cancer, targeting malignant cells and providing a precise immune response through tumor antigen recognition[1].

There are different types of immunotherapy, so different types of cancer responses can be achieved. All of them are bound by a fundamental principle: Immunotherapy is different from standard therapies (*i.e.* chemotherapy, radiotherapy, or oncologic surgery) because it helps the self-response to cancer[2].

For these reasons, the standard criteria for monitoring the success of therapy in oncologic patients are not sufficient. All scores, including the World Health Organization classification and the response evaluation criteria in solid tumors (RECIST 1.1.), do not consider that fighting cancer for immunotherapy requires a synergy between tumor cells and host cells[3,4]. To obviate this essential issue, since 2004, different criteria were developed to analyze these responses such as immune-related response criteria, immune-related RECIST, and finally in 2017 immuno-RECIST (iRECIST)[5-8]. These new criteria aim to consider the variety and the time of response to immunotherapy compared with standard therapy, and to standardize and validate the radiologic and clinical interpretation[9].

However, immunotherapy raises different questions such as: why is the target lesion increased at first control after immunotherapy and reduced at its end? Why is the target legion bigger at the end of treatment, but the patient's conditions improve? Why do some metastases disappear, and others become bigger? These different phenomena are called pseudoprogression, hyperprogression, and dissociate response, respectively, and belong to the new lexicon of cancer response to immunotherapeutic agents[10,11].

Radiologists and clinicians should be confident with these patterns (Figure 1) and the interpretation of these data to better understand and manage oncologic patients who have undergone immunotherapy.

In this setting, the present review aims to critically analyze and summarize the most common type of responses to immunotherapy and to drive the knowledge of correct radiologic and clinical interpretation of iRECIST, strengthening the communication skills between specialists.

PSEUDOPROGRESSION

Pseudoprogression is defined as the phenomenon characterized by an initial increase in primary tumor size or new lesions appearance, after starting immunotherapy, followed by a decrease in tumor burden[12-15]. Pseudoprogression should not be considered a true tumor progression but an infiltration and recruitment of various immune cells, such as T or B lymphocytes in the tumor core[16]. Two biological hypotheses have been proposed to explain the phenomenon of pseudoprogression

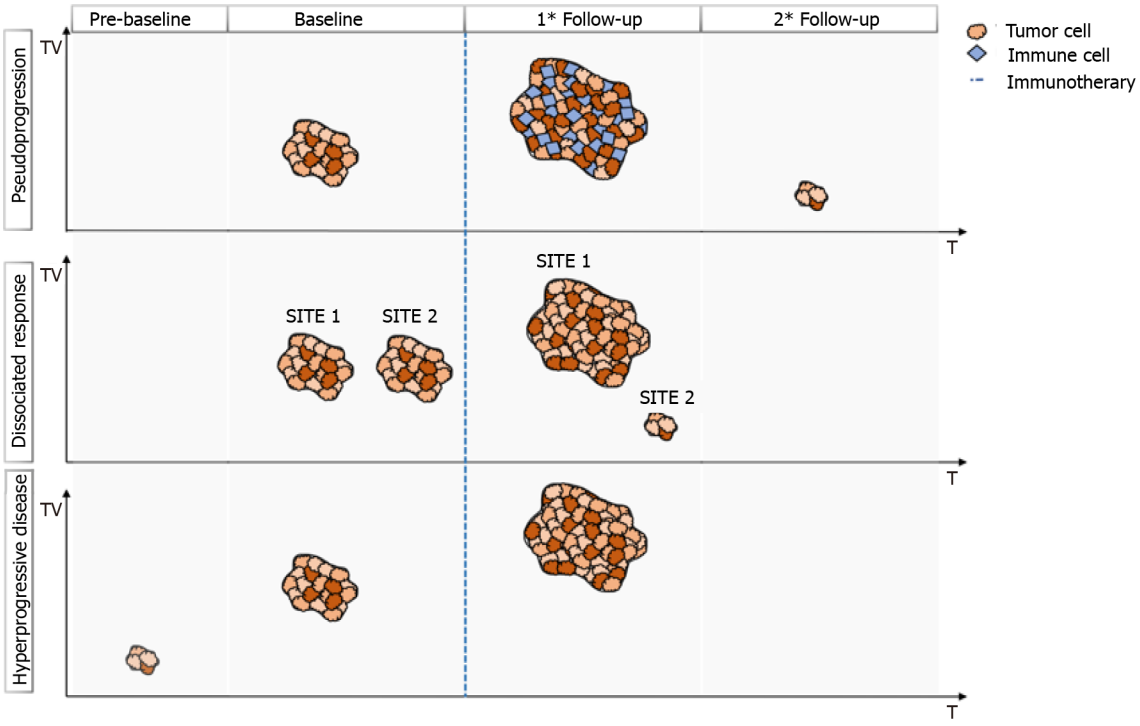


Figure 1 Graphical summary of different responses to immunotherapy. Pseudoprogression: increase of longest diameter > 20% at first follow-up, followed by a decrease of > 30% at subsequent follow-up. Dissociated response: increase of some target lesions of > 20% and reduction of at least another target lesion > 30% at follow-up. Hyperprogression: significant increase of target lesion at first follow-up; a baseline study is needed to correctly assess the hyperprogression. T: Time; TV: Tumor volume.

observed in patients treated with immuno-oncology agents. The first hypothesis concerns tumors' continuous growth until the activation of an effective antitumoral immune response; the second one suggests that an immune-cell influx could occur in the tumoral microenvironment caused by the reactivation of the immune system, leading to inflammation and a transient increase of tumor burden[15].

A study by Cohen *et al*[17] described the case of a patient with melanoma brain metastasis, who was treated with pembrolizumab, presenting a pseudoprogression of brain lesions revealed through magnetic resonance imaging (MRI) and biopsy. The MRI showed an enlargement of central nervous system lesions with diffuse perilesional edema, while the histologic evaluation revealed tumor cells surrounded by reactive astrocytosis, scattered inflammatory cells, and microglial cells, which was consistent with the abovementioned response to treatment rather than tumor growth.

Rocha *et al*[18] described the case of a patient with end-stage squamous cell lung cancer, who was treated with nivolumab and exhibited pseudoprogression of the liver lesions, proved by the biopsy. The tissue sample revealed extensive areas of necrosis, no viable tumor cells, and lymphocyte infiltration. In the liver biopsy, the number of $CD4^-$, $CD8^-$ and $CD103^-$ cells were increased, the ratio of $CD4^+/CD8^+$ T cells was decreased, and $CD68^+$ staining indicated a higher proportion of macrophages, suggesting an inflammatory response rather than disease progression. Moreover, other cases have shown necrosis, hemorrhage, edema, and immune cell infiltration in lesions with pseudoprogression[14,15,19,20]. Therefore, the infiltration of immune cells, such as $CD4^+$, $CD8^+$ cells and macrophages, represents the major mechanism of pseudoprogression, consequently including edema, hemorrhage, and necrosis[12].

An unconventional pattern of response to immunotherapy was first described with the development of cytotoxic T-lymphocyte antigen 4 inhibitors in melanoma, with a patient experiencing enlargement of a cutaneous lesion during the first weeks of treatment, followed by prolonged stabilization[15]. Since then, pseudoprogression has been used to describe an objective response obtained after initial progression disease and has been observed in other cancer types[16] (Figure 2).

The occurrence of pseudoprogression was confirmed in large trials, which allows treatment beyond progression; its incidence, reported in different tumor types, has never exceeded 10% of patients[21]. However, a recent study determined that the incidence of atypical response is about 20%, including the development of new lesions, and the increase greater than 10% in the total sum of the longest dimension[22].

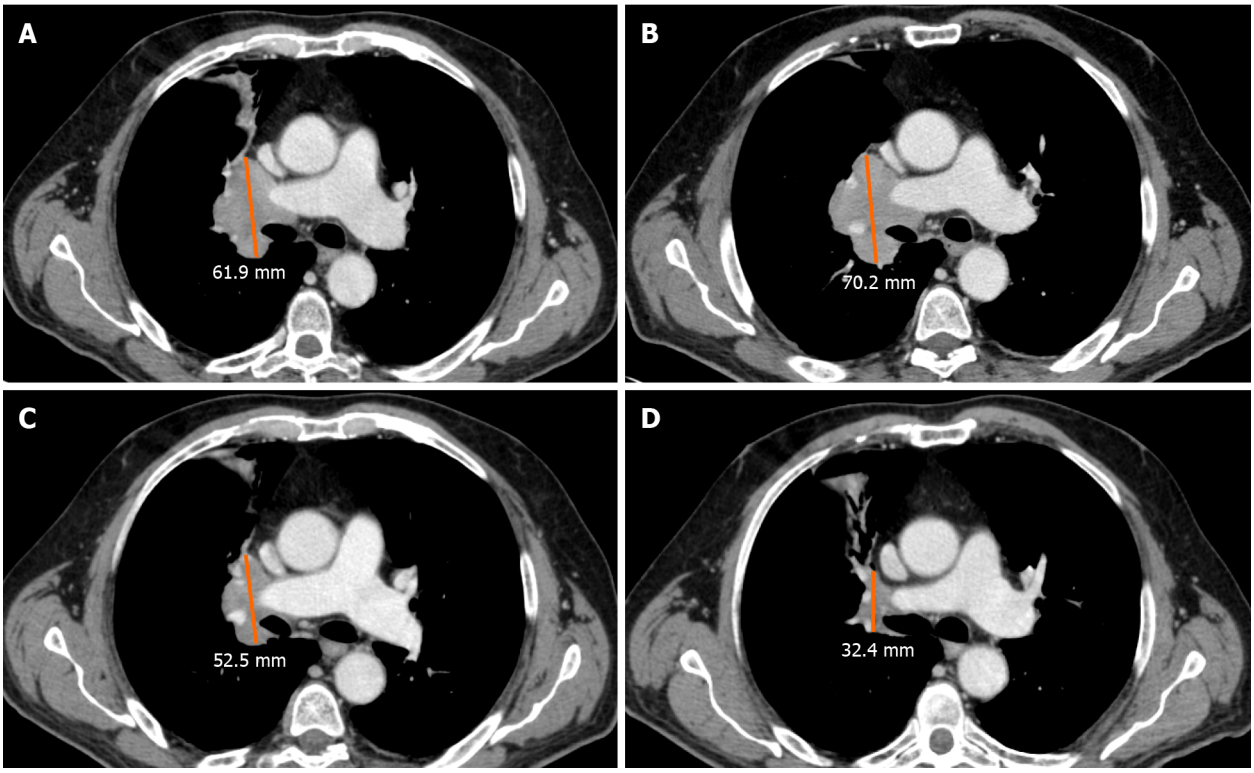


Figure 2 Axial computed tomography images in the portal-venous phase of a 69 y/o male, ex-smoker with non-small lung cell carcinoma, during second-line therapy with Atezolizumab. A: Pre-treatment imaging show the right peri-hilar lesion; B: During follow-up after 4 wk the lesion increase in size; C and D: During the following computed tomography scans (8 and 12 wk) a significant decrease in longest diameter was achieved, confirming a final response to treatment with the presence of intercurrent (B) pseudoprogression.

Pseudoprogression has been described in different types of tumors, mainly in melanoma patients but also in non-small lung cell carcinoma (NSCLC) (Figure 3), renal cancer (RCC), urothelial cancer, uveal melanoma, Merkel cell carcinoma, mesothelioma, Hodgkin lymphoma, and head and neck squamous cell carcinoma (HNSCC) [23] and it can also occur in metastatic lesions and some oncologic patients with pleural effusion and ascites[14,24-29].

The reported incidence of pseudoprogression in clinical trials was 2.78%-9.69% for melanoma, 1.81%-5.77% for NSCLC, 2.86%-8.82% for RCC, 1.49%-7.14% for urothelial carcinoma, 11.11% for uveal melanoma, 1.79% for HNSCC, 1.14% for Merkel cell carcinoma, and 6.90% for mesothelioma[12].

Clinical and biological characteristics of different tumors, the demographic characteristics of patients, and the different types of immunotherapy agents used might explain the different incidence of pseudoprogression in various types of solid tumors. In addition, according to some case reports, there might be some sites of pseudoprogression specific to the tumor type after immunotherapy, such as brain metastasis pseudoprogression of lung cancer and RCC[30,31].

Interestingly, for patients treated beyond progression, no increase in immune-related toxicity was reported. Furthermore, patients experiencing pseudoprogression had longer overall survival (OS) compared with standard progressive disease (PD), suggesting that patients who present with pseudoprogression can effectively obtain benefit from treatment beyond progression[23].

The iRECIST guidelines proposed two specific response patterns: unconfirmed PD (iUPD) and confirmed PD (iCPD). The iUPD is defined as PD for the RECIST v1.1 criteria that is not confirmed at the follow-up imaging assessment within 4-8 wk. The iCPD is defined as the appearance of a new lesion or further growth of the sum of measures of target lesions of 5 mm or greater at the diagnostic follow-up after the iUPD within 4-8 wk, or as an increase in a non-target lesion, that was initially categorized as iUPD. If no change in tumor size nor extent from iUPD occurs, then the time point response would again be iUPD. Complete response (iCR), partial response (iPR), and stable disease (iSD) were assigned based on the RECIST 1.1. Moreover, if after iSD, iPR, or iCR, PD takes place again, we consider it as iUPD and reset the bar again through the application of the so-called "dynamic time point"[7]. To resume, iUPD can be assigned multiple times as long as iCPD is not confirmed at the next

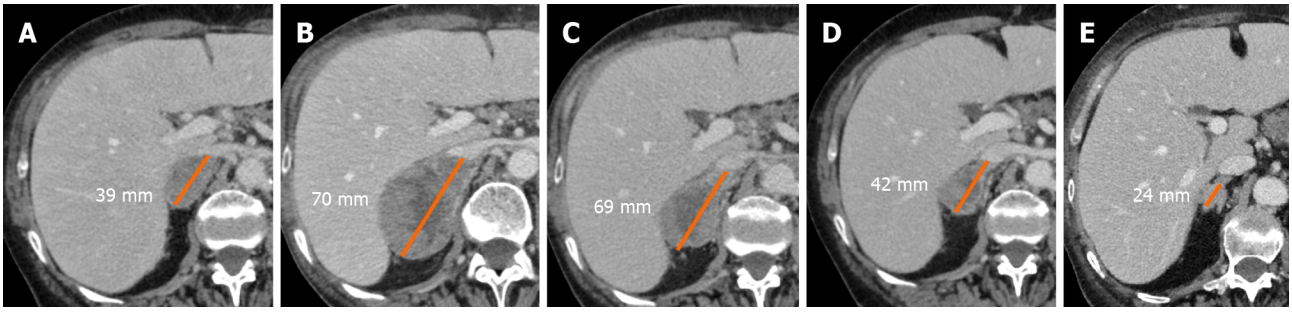


Figure 3 Axial computed tomography images in the portal-venous phase of a 65 y/o female, with non-small lung cell carcinoma and a programmed death-ligand 1 expression > 90%, during first-line treatment with Pembrolizumab. A: In the baseline examination, computed tomography (CT) shows a metastatic lesion in the right adrenal gland; B: After 4 wk of treatment, the lesion becomes bigger, with a total increment of 21%, referred to as unconfirmed progression; C-E: during following CT scans, the target lesion shows a progressive dimensional reduction with a total decrease of 46%. These variations in the size of the lesion, during immunotherapy, was in line with pseudoprogression.

assessment and iRECIST requires the confirmation of progression to rule out or confirm pseudoprogression.

The iRECIST guidelines proposed a status of iUPD, which would allow the continuation of treatment and follow-up more closely to better benefit patients. This approach allows the identification, understanding, and better characterization of atypical responses, such as delayed responses that occur after pseudoprogression[7].

To differentiate pseudoprogression from true progression, the iRECIST guidelines recommend that clinical trials should only include patients who are clinically stable to continue treatments until the next assessment (≥ 4 wk later). In these cases, the next imaging assessment should be performed no longer than 8 wk later, to ensure that patients remain fit for rescue therapies[7].

Among the potential useful methods to identify pseudoprogression in tumors treated with immunotherapy and to differentiate it from the true progression of the disease, the combination of biopsy and histopathologic examination is considered the gold standard, although it presents some disadvantages due to the invasive nature of the procedure. Compared to biopsy, the radiographic follow-up presents incomparable advantages in the monitoring of pseudoprogression. It could be used in any type of tumor with measurable lesions and it is convenient, non-invasive, and can avoid prematurely discontinued immunotherapy for pseudoprogression. In addition to radiological computed tomography (CT) follow-up, other proposed methods to determine pseudoprogression included MRI and positron emission tomography (PET)/CT, which can distinguish inflammatory cell infiltration from the enlarged tumor tissue, at least theoretically. In recent literature, the circulating tumor's DNA and interleukin-8 serum levels were purposed in the follow-up of oncologic patients to quickly identify a possible pseudoprogression[12].

A summary of the most important studies focusing on pseudoprogression is reported in Table 1.

HYPERPROGRESSION

Hyperprogressive disease (HPD) is considered fast tumor growth, after starting immunotherapy, regarding the absolute mass. However, compared with the other atypical patterns, HPD relies on its intrinsic definition in the "expected" response, and consequently, a specific description is currently missing. For example, empiric doubling of tumor volume or by using linear growth in tumor diameter have been proposed to identify the HPD and, as a matter of fact, recently published papers reported different ways to define HPD and different thresholds to stratify pa-tients[32-34]. Moreover, considering that HPD can be shown in different cancer types, a standardized definition is needed.

Different cellular and genetic triggering events were studied to better define and understand HPD. The first described is linked to cytotoxic agents used before immunotherapy, probably causing a decreased effect of the last one[35] due to clones' selection able to escape therapy. On the other hand, new immunotherapeutic agents can bind other than targeted receptors and allow rapid tumor growth. Finally, different genetic mutations, such as the most common one Janus kinase 1/2 mutation,

Table 1 Incidence of atypical response in different cancer types and treatments, according to the most recent literature

Response	Cancer type	Treatment	Incidence (% range)
Pseudoprogression	Melanoma	Ipilimumab	7.4-9.7
		Tremelimumab	2.8-6.3
		PD1/PD-L1 inhibitors	3.7-8.3
		Pembrolizumab	3.7-7.3
	RCC	PD1/PD-L1 inhibitors	4.9-14.8
		Atezolizumab	2.9
	NSCLC	PD1/PD-L1 inhibitors	1.9-6.9
		Atezolizumab	2.8
	Urothelial	Atezolizumab	1.5-6.8
		Durvalumab	7.1
		PD1/PD-L1 inhibitors	8.9
	HNSCC	Pembrolizumab	1.8
		PD1/PD-L1 inhibitors	1.3
	Mesothelioma	Tremelimumab	6.9
Hyperprogression	NSCLC	PD1/PD-L1 inhibitors	8.0-14.0
	Gastric	PD1/PD-L1 inhibitors	21.0-29.4
	RCC	PD1/PD-L1 inhibitors	7.0-46.0
	Melanoma	PD1/PD-L1 inhibitors	1.2
Dissociated response	NSCLC	PD1/PD-L1 inhibitors	7-5-10

HNSCC: Head and neck squamous cell carcinoma; NSCLC: Non-small lung cell carcinoma; PD1: Programmed cell death 1; PD-L1: Programmed death-ligand 1; RCC: Renal cell carcinoma.

can be directly linked to HPD, generating resistance to immunotherapy and resulting in a fast tumor volume increase. The tumor microenvironment can be strictly involved in HPD, especially by immune cell infiltration, as reported in previous papers[36-38].

From a radiological point of view, to identify HPD, at least one imaging exam should be obtained before and one after starting immunotherapy, to correctly establish an increase in tumor volume higher than the expected one[39,40].

Even if the iRECIST algorithm is the most widely applied in clinical practice, it does not suggest evaluating the pretreatment imaging data to identify the tumor growth rate (TGR), and suspected hyperprogressive patients should be followed-up for at least 12 wk for definitive confirmation[39]. The identification of HPD poses a challenge for the iRECIST, which fail to capture pre- and post-treatment tumor growth kinetics (TGK) at early times of disease, and consequently, different parameters such as "RECIST progression at the first evaluation"[39], TGR[40], TGK ratio (ratio of the slope of tumor growth before treatment and the slope of tumor growth on treatment), time to treatment failure (TTF)[41], and the combination of clinical and radiological criteria [42] have been proposed.

A recent study by Gomes da Morais *et al*[43], combining four different definitions for HPD previously proposed, found no overall significant differences between baseline and post-baseline tumor growth rate (P = 0.93). Finally, the authors confirmed that the progression-free survival (PFS) was shorter in patients with HPD compared with non-HPD ones.

A metanalysis published by Kim *et al*[44] evaluated a total of 217 HPD cases of 1519 cancer patients. Considering the lack in HPD definition, its incidence ranged from 1% to 30%, in line with Frelaut *et al*[23], reporting a range from 7% and 29%. Authors identified age (> 65 years), gender (female), aggressive primary tumor (high recurrence rate, > 2 metastatic sites), histological and immunological profiling (*i.e.* low programmed death-ligand 1 expression, epidermal growth factor receptor, mouse double minute 2 homology and DNA (cytosine-5)-methyltransferase 3A alterations) as predictive factors for HPD.

Analyzing the most important recent studies, Park *et al*[45] identified HPD in 18 patients (14.4%) with head and neck cancer, underlying that younger age, a primary tumor of the oral cavity, and previous locoregional irradiation are significant predictors of HPD. Moreover, patients with HPD showed a shorter median PFS and OS.

To date, different published papers have investigated the importance of HPD in lung cancer patients. Kim *et al*[46] observed HPD in 55 (20.9%), 54 (20.5%), and 98 (37.3%) patients according to the TGK, TGR, and TTF, underlying that HPD was associated with worse PFS and OS. The same results in terms of incidence were reported in previous retrospective studies by Ferrara *et al*[40] (14%), Lo Russo *et al*[42] (26%), Kim *et al*[46] (21%), as summarized in the review by Kim *et al*[44]. More recently, Kas *et al*[47], with a retrospective study including 406 patients, suggested a new definition for HPD in patients with NSCLC, based on ΔTGR.

Aoki *et al*[48] and Sasaki *et al*[49] studied the importance of HPD in gastric cancer patients reporting an incidence of 29.4% and 21% after nivolumab treatment, respectively. Both studies reported a slight decrease in PFS and OS in patients with HPD.

Kim *et al*[50] reported that HPD exists in a fraction of hepatocellular carcinoma (HCC) patients who received programmed cell death protein 1 (PD-1) blockade: Analyses of the baseline immune profile and on-treatment tumor growth dynamics could promote optimal patient selection and earlier identification of rapid tumor growth induced by PD-1 inhibitors in HCC patients[50].

Zheng *et al*[51] reviewed patients with RCC under immunotherapy, finding that the incidence of HPD ranged between 7% and 74% without any strong suggestive factors associated.

Regarding melanoma, immunotherapy treatment is not extensively reported in the literature. A recent retrospective study by Hao *et al*[52] and Schuiveling *et al*[53], enrolling 168 patients, reported a 1.2% incidence of HPD.

According to the RECIST working group, a CT scan 8 wk after the first treatment is needed to evaluate early response[7]. In line with the guidelines, if progression is not confirmed, the follow-up should be continued as previously planned, while in case of suspected progression at first-imaging follow-up, a confirmatory CT 4 wk later should be required. Moreover, considering the importance of pre-baseline imaging, a CT scan at least 1 mo before starting immunotherapy should be evaluated to define the tumor volume and consider it in further evaluations. During the anamnestic questionnaire, special attention should be addressed to pre-immunotherapy treatments, specifically regarding conventional cytotoxic agents, as aforementioned[39,40]. Radiological assessment, both CT- and MRI-based, is fundamental to determine the growth rate; however, the true positive rate can be weakened by pseudoprogression in case of pre-baseline missing, because it is not possible to distinguish between the two patterns.

On these bases, a complete assessment based on clinical and radiological findings, along with a careful evaluation of pre-baseline imaging, is needed to correctly stratify patients suspected of HPD, to define the best clinical approach possible to increase PFS and OS. The difficulties to standardize the HPD definition by using radiological criteria firstly rely on the various types of cancer to deal with and, consequently, on the different imaging techniques considered as the reference standard for staging and re-staging patients.

A summary of the most important studies focusing on hyperprogression is reported in Table 1.

DISSOCIATED RESPONSE

Besides the mixed pattern of response arising from traditional platinum-based chemotherapy, the development of immunotherapy has led to the introduction of the concept of dissociated response (DR).

DR has been recently described as a concomitant increase in the size of some target lesions or the appearance of new lesions, accompanied by regression of other ones[54]. A combination of factors may explain the biological mechanisms of a dissociated tumor response. Tumor heterogeneity within an individual patient and differences in tissue penetration of anti-cancer drugs have been proposed as potential reasons for DR [55]. Tumoral cells can undergo clonal evolution from a single progenitor cell into more aggressive and therapy-resistant cells, due to genomic instability of solid cancer cells.

This genotypic and phenotypic heterogeneity is an unfavorable prognostic factor for cells' survival, and it can explain the DR, particularly when using targeted therapies due to their selective pressure on tumor evolution. Moreover, the heterogeneity of the immune environment of the lesions can actively influence therapeutic response and therefore explain different responses[56] (Figure 4).

In literature few studies reported on the incidence of DR, ranging from 7.5% to 10% [54,55,57]. Using fluorodeoxyglucose PET/CT, Humbert *et al*[58] recently pub-lished a prospective study including 50 patients with NSCLC treated with pembrolizumab in first-line therapy or with nivolumab in second-line therapy, showing that 10% of the population had a DR.

DR has been associated with different prognoses compared to progressive or non-PD. Tazdait *et al*[54] observed similar survival between patients with the non-PD and those with the atypical response, even if pseudoprogression and DR were not evaluated separately. On the contrary, the higher survival of patients with DR, compared to those with PD, was confirmed both by Tazdait *et al*[54] and Tozuka *et al* [55], suggesting that the prognosis of patients with DR is probably intermediate between those with PD and those with the non-PD.

In the literature, several different definitions of DR were encountered; in particular, it is still not clear if a concomitant progression and reduction of different lesions are sufficient to consider as DR, or if it is necessary to reach at least 20% of PD and 30% of PR[54,55,57]. On PET/CT, DR definition should be inspired by PET Response Criteria In solid tumor (PERCIST) and defined as a concomitant relative decrease > 30% in some tumor lesions metabolism and relative metabolic increase > 30% in others.

An important issue is the optimal duration of treatment due to the potential of late treatment effect and the rare phenomenon of pseudoprogression. Many clinicians choose to continue treatment beyond progression with immunotherapy according to the RECIST[59]. As the progressing lesions might represent pseudoprogression, the monitoring and management of patients with the DR should be similar to that of patients with pseudoprogression, if the patient is clinically stable. A recent study shows that continuing immunotherapy post-DR had significantly better survival than discontinuing therapy[57].

Besides, continuing immune checkpoint inhibitor treatment plus local ablative therapy targeted to progressing lesions could be a valid alternative to immunotherapy alone in case of single progressive lesions[56]. However, if the patient is clinically deteriorating the interruption of immune checkpoint inhibitor treatment and switching to another therapy, or clinical trial participation, should be considered[60].

The high number of atypical responses such as pseudoprogression and DR suggest that in most cases the RECIST 1.1 underestimates the benefit of treatment with immunotherapy and the new iRECIST are certainly superior in the evaluation of responses. The iRECIST consider consistently pseudoprogression, while DR is not considered[7,11], suggesting that they may not correctly describe the clinical benefit from immunotherapy[61].

Considering the different interpretations of DR given by the different authors, a more uniform definition of this phenomenon is crucial to assess the correct prognosis of patients with DR compared to progressive and non-PD after immunotherapy. As suggested by Humbert and Chardin[56], DR on CT exam should be inspired by RECIST 1.1, defined as a concomitant decrease in size > 30% in some lesions and increase in size > 20% in others (and/or presence of new lesions), while on PET/CT, DR should be motivated by PERCIST criteria, defined as a concomitant decrease > 30% in some tumor lesions metabolism and metabolic increase > 30% in others (and/or new hypermetabolic lesions).

A summary of the most important studies focusing on DR is reported in Table 1.

To conclude, DR should be considered in the iRECIST in addition to or separately from a PD, partial response, and stable disease, through radiological evaluation, for a more precise evaluation of tumor response to the immunotherapy.

CONCLUSION

iRECIST can help to correctly categorize the classes of response to immunotherapy treatment by dividing patients into four main groups (iSD, iPR, iCR, iPD), according to the radiological target lesion modifications, achieved along the time, and the standard solid response criteria (RECIST 1.1). Recently, other different kinds of response have been described in literature after immunotherapy treatment, defined as atypical responses, categorized in three patterns: pseudoprogression, hyperprogression, and

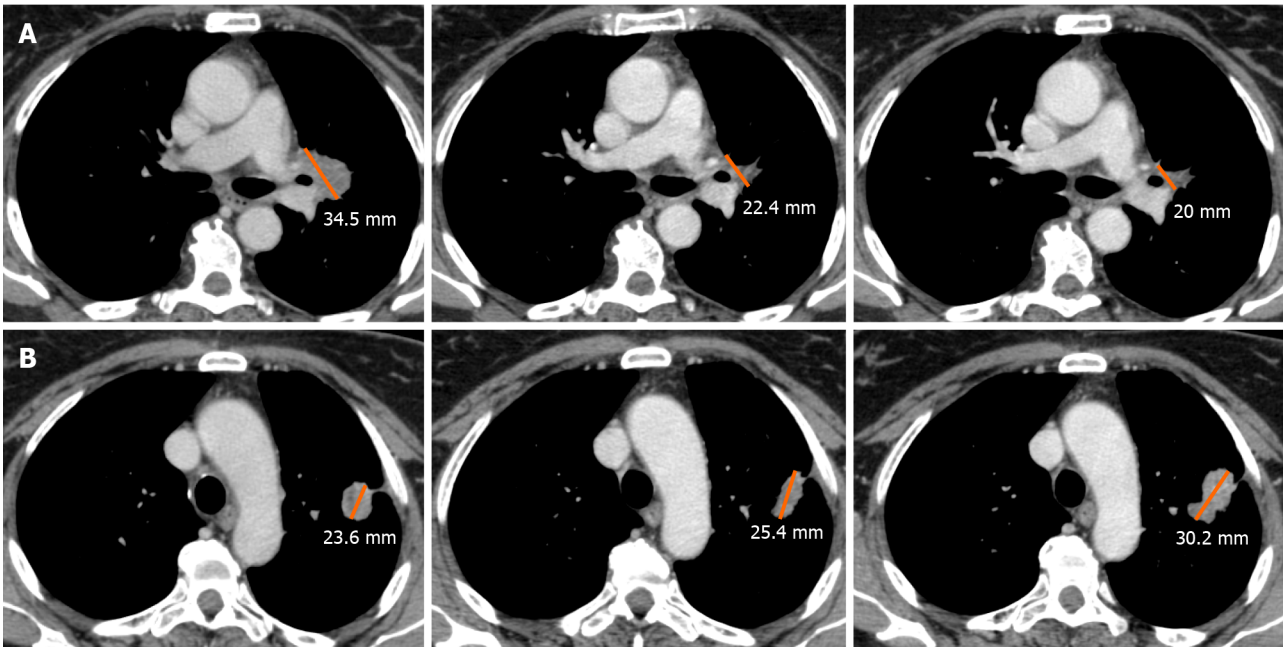


Figure 4 Axial computed tomography images in the portal-venous phase of a 57 y/o female ex-smoker with non-small lung cell carcinoma during second-line treatment with Pembrolizumab. Images show a dissociated response of two target lesions. A: The left peri-hilar lesion progressively decreased in size during follow-up, if compared to the pre-treatment computed tomography scan (after 3 wk and after 9 wk of immunotherapy from left to right, respectively); B: The second target lesion in left lung firstly regressed after 3 wk of immunotherapy showing, then a progression during the follow-up period (from left to right, respectively).

dissociated response. The correct knowledge of these new atypical patterns should be correctly assessed by both radiologists and clinicians, through the deep investigation of clinical anamnesis and imaging findings to guarantee the best management.

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MINIREVIEWS

Hepatic Langerhans cell histiocytosis: A review

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Abstract

Hepatic Langerhans cell histiocytosis (LCH) is characterized by proliferation and accumulation of Langerhans cells in the liver, causing liver dysfunction or forming a mass lesion. The liver can be involved in isolation, or be affected along with other organs. A common clinical hepatic presentation is cholestasis with pruritis, fatigue and direct hyperbilirubinemia. In late stages, there may be hypoalbuminemia. Liver biopsy may be required for the diagnosis of hepatic LCH. Histologic finding may be diverse, including lobular Langerhans cell infiltrate with mixed inflammatory background, primary biliary cholangitis-like pattern, sclerosing cholangitis-like pattern, and even cirrhosis at later stages. Because of its non-specific injury patterns with broad differential diagnosis, establishing a diagnosis of hepatic LCH can be challenging. Hepatic LCH can easily be missed unless this diagnosis is considered at the time of biopsy interpretation. A definitive diagnosis relies on positive staining with CD1a and S100 antigen. Liver involvement is a high risk feature in LCH. The overall prognosis of hepatic LCH is poor. Treating at an early stage may improve the outcome. Systemic chemotherapy is the mainstay of treatment and liver transplantation may be offered. New molecular markers involved in pathogenesis of LCH are being explored with a potential for targeted therapy. However, further studies are needed to improve outcome.

Key Words: Langerhans cell; Liver; Cholangitis; CD1a

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Core Tip: Hepatic Langerhans cell histiocytosis (LCH) is characterized by proliferation and accumulation of Langerhans cells in the liver, causing liver dysfunction or forming

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a mass lesion. There are diverse injury patterns on liver biopsy, including lobular Langerhans cell infiltrate with mixed inflammatory background, primary biliary cholangitis-like pattern, sclerosing cholangitis-like pattern, and even cirrhosis at later stage. The overall prognosis of hepatic LCH is poor. New molecular markers involved in pathogenesis of LCH are being explored with a potential for targeted therapy.

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INTRODUCTION

Langerhans cell histiocytosis (LCH) is characterized by proliferation and accumulation of dendritic antigen-presenting histiocytes, Langerhans cells, in tissue. Langerhans cells express CD1a, S-100 and langerin proteins, and show Birbeck granules on ultrastructural examination[1]. LCH is a rare disease with an annual incidence of about 5 cases *per* 1 million population. The disease is more common in Caucasian population of northern European descent, with a male predominance. It can affect any age group but most cases occur in children[1,2]. The etiology is unknown. However, there is a strong association between pulmonary LCH and smoking[3,4].

Some authors have considered that LCH is an abnormal reactive process to an inciting event, such as viral infection, given the fact that multiple cytokines are involved in the process and LCH has been reported to regress spontaneously when it's an isolated lesion[5,6]. However, the revised 4th edition World Health Organization[1] classified LCH as a clonal neoplastic process. Recently, Murakami *et al*[7] proposed that both *BRAF* mutation and Interleukin-1 Loop amplification play important roles in the pathogenesis of LCH[7]. More recently, other groups found that *BRAF*V600E mutation or alternative activating MAPK pathway gene mutations are almost universally identified in LCH[8,9]. These studies provide a potential for molecular alteration-based targeted therapy for LCH.

LCH demonstrates a variable clinical picture and course. It can involve a single organ system (SS-LCH) or multiple organ systems (MS-LCH). While any organ can be affected, bone is the most frequent site occurring in 80% of cases of LCH; it can be unifocal or multifocal. A third of LCH cases involve the skin and a quarter involve the pituitary gland. Other organs, such as liver, spleen, lungs, lymph nodes, gastrointestinal tract, and rarely parotid glands and nails, can also be involved[10].

When LCH involves a single organ, the course may be indolent and have a favorable survival. Su *et al*[11] reported 100% 5-year survival in pediatric LCH patients when the disease involved only bone[11]. In a long-term (over 17 years) follow-up study of pediatric LCH, SS-LCH showed a 100% regression rate and low relapse *vs* 73% regression in MS-LCH[12].

LCH involving the skin, bone, lymph nodes or pituitary gland is considered "low-risk" because of its good response to treatment. Involvement of the lungs, hematopoietic system and spleen, and liver is considered "high risk" with an unfavorable outcome[1,6,12,13].

In this review, we focus on hepatic LCH and provide a brief overview of its clinical presentation, laboratory/imaging findings and current treatment. Its pathologic findings and differential diagnosis are also reviewed.

DIAGNOSIS OF HEPATIC LCH

The diagnosis of hepatic LCH in a patient with known LCH requires one or more of the followings: (1) hepatomegaly, defined as a liver edge greater than 3 cm below the costal margin at the mid clavicular line (confirmed by ultrasound); (2) liver dysfunction defined either by abnormal serum biochemical tests including bilirubin greater than 3 times the upper limit of normal, protein less than 55 g/L, albumin less than 3 g/dL, transaminases [alanine aminotransferase (ALT)/ aspartate aminotransferase (AST)] greater than 3 times normal, or by clinical entities including an

intrahepatic nodular mass or ascites or edema, not as a result of other causes; or (3) histopathological findings of active disease[14,15].

INCIDENCE OF HEPATIC LCH

The liver may be affected in isolation[16-19], or involved along with other organ systems such as lymph nodes, skin and lungs.

In pediatric LCH, the frequency of liver involvement is variable but may be high (15%-60%). It carries a poor prognosis. In adult LCH, liver involvement is relatively uncommon with an incidence of 16%-27%. However, it is probably under recognized. One study of multisystemic LCH in adults reported liver involvement in 87%[14,18,20].

CLINICAL MANIFESTATION

There are 2 stages of hepatic LCH: An early stage with liver parenchymal infiltration by Langerhans cells and a late stage with biliary sclerosis[18,20]. Clinical manifestations differ based on the stage.

A common presentation is jaundice with direct hyperbilirubinemia and hypoalbuminemia. Patients may present with fatigue and pruritus[21]. Clinically, hepatic LCH may mimic other conditions such as chronic destructive cholangitis, metabolic disease, hepatitis, neoplasia obstructing biliary tract and inherited deficient conjugation of bilirubin. It can also present as Reye syndrome, chronic inflammatory bowel disease, or hemochromatosis[14]. In late stages, patients may present with severe sclerosing cholangitis and liver failure. A subset of patients progresses to develop cirrhosis, which may lead to portal hypertension and secondary hypersplenism[22,23].

LABORATORY/IMAGE FINDINGS

Laboratory tests show abnormal serum liver tests with mild to moderately elevated ALT and AST. Cholestatic biochemical profiles are seen with increased total bilirubin, γ glutamyl transferases and anaplastic lymphoma kinase phosphatase. The albumin level is often low. The prothrombin time may be prolonged due to decreased clotting factor. Depending on liver function, clotting factors may be depleted. Complete blood count is usually normal, although the platelet count may be low in patients with portal hypertension and splenomegaly. Abdominal contrast-enhanced computed tomography may demonstrate hepatomegaly with solitary or multiple hypodense hepatic nodules, which can be confluent[18]. At a late stage, magnetic resonance imaging may show biliary tree abnormalities[20].

PATHOLOGY FINDINGS

Liver biopsy is the cornerstone of the diagnosis of LCH and disease staging. Liver biopsy can show various injury patterns. In an early stage, lobular Langerhans cell infiltrate mixed with lymphocytes can be seen. The Langerhans cells may form focal aggregates or be multinucleated[18]. In addition to lymphocytes, mature eosinophils, neutrophils and plasma cells can also be noted. A definitive diagnosis can be rendered based on positive immunohistochemical staining with CD1a and S100 antigen of the Langerhans cells.

At a later stage, Langerhans cells may infiltrate the bile ducts and cause sclerosing cholangitis. In some cases, histologic features of sclerosing cholangitis may be seen in a biopsy without identifiable Langerhans cells[17,22,24]. This may be due to selective involvement of the major bile ducts by Langerhans cells; large ducts are unlikely to be sampled in the usual needle biopsy[25]. Chronic non-suppurative destructive cholangitis injury pattern has been also reported[26].

Notably, hepatic LCH may mimic primary biliary cholangitis (PBC). Rush *et al*[19] reported hepatic LCH presenting as a small noncaseating granuloma in the portal tract with rare multinucleated epithelioid giant cells within the portal inflammatory infiltrate. The patient underwent a liver transplantation for the presumed diagnosis of advanced anti-mitochondrial antibody (AMA)-negative PBC. However, the disease

recurred in the allograft. The diagnosis of hepatic LCH was finally rendered three years after transplantation in the allograft biopsy. Four years later (7 years after the transplantation), the patient lost the liver graft. LCH diagnosis was confirmed in the explanted allograft liver[19].

Similarly, our group reported a case of hepatic LCH that histologically and pathologically mimicked PBC. A 65-year-old man presented with intermittent pruritus with cholestatic biochemistry profile for years. The liver biopsy showed portal histiocytic aggregate (non-necrotizing granuloma) encasing a damaged bile duct (Figure 1). Tests for autoantibodies including AMA were negative, therefore AMA-negative PBC was suspected. One month later, multiple skin lesions developed and a diagnosis of LCH was rendered on a skin biopsy. In light of the positive skin biopsy, the previous liver biopsy was re-examined. The histiocytes surrounding the duct were positive for CD1a and S100, confirming the diagnosis of hepatic LCH, retrospectively[21].

These two cases highlight the difficulty of rendering a diagnosis of hepatic LCH without appropriate clinical context.

In summary, hepatic LCH may present as a non-specific inflammatory process with varying injury pattern. Therefore, the diagnosis of hepatic LCH can easily be missed when this diagnosis is not considered. In addition, its patchy nature and limited sampling may further hinder the diagnosis of hepatic LCH[21]. In late stage MS-LCH, even though the clinical picture may be strongly indicative of hepatic LCH, it may be difficult to render a definitive diagnosis on the initial biopsy and a repeat biopsy may be necessary[6].

DIFFERENTIAL DIAGNOSIS

There are a variety of differential diagnoses for hepatic LCH due to its nonspecific presentations and morphologic features.

The morphological and clinical findings of hepatic LCH may resemble primary sclerosing cholangitis (PSC). A classic "beaded" appearance of extrahepatic biliary tree by endoscopic retrograde cholangiopancreatography and periductal onion-skin type fibrosis with negative stains for S100 and CD1a may be helpful in the diagnosis of PSC.

Granulomatous reaction with ductular proliferation raises a differential diagnosis of PBC. The presence of a dense portal lymphoplasmacytic infiltrate, mild degree of interface activity, and negativity for S100 and CD1a in conjunction with positivity for AMA can be helpful for PBC diagnosis. However, as demonstrated above, it can be very difficult to differentiate hepatic LCH from AMA negative PBC in a small liver biopsy, especially in the absence of appropriate clinical context.

In hepatic LCH, the presence of granulomatous inflammation also raises a differential diagnosis of infection. Grocott (or Gomori) methenamine silver and acid-fast bacteria stains may help to rule out infectious etiology, although they have low sensitivity[27-30]. Tissue culture may be helpful in these scenarios. Other differential diagnosis includes sarcoidosis, foreign-body type giant cell reactions, drug-induced liver injury, and non-Hodgkin's and Hodgkin's lymphoma.

Additionally, myeloproliferative disorders and myeloid leukemias can express CD1a and/or S100 protein, mimicking LCH. However, these are distinguished by sinusoidal infiltrative pattern of the neoplastic cells[25].

TREATMENT

Depending on clinical course and degree of organ dysfunction, variable treatment options are offered for LCH. Usually, patients with single site SS-LCH are observed only or offered monotherapy, such as oral 6-mercaptopurine and methotrexate, indomethacin, bisphosphonates, and hydroxyurea. Patients with MS-LCH usually benefit from systemic therapy, such as the vinblastine-prednisone combination [6,15, 20].

NOVEL MOLECULAR TARGETS

Recently, molecular markers such as *BRAFV600E* and *MAP2K1* have been identified in LCH pathogenesis. These markers may serve as molecular targets for precision therapy in the future. For instance, vemurafenib, a *BRAF* inhibitor, had prolonged

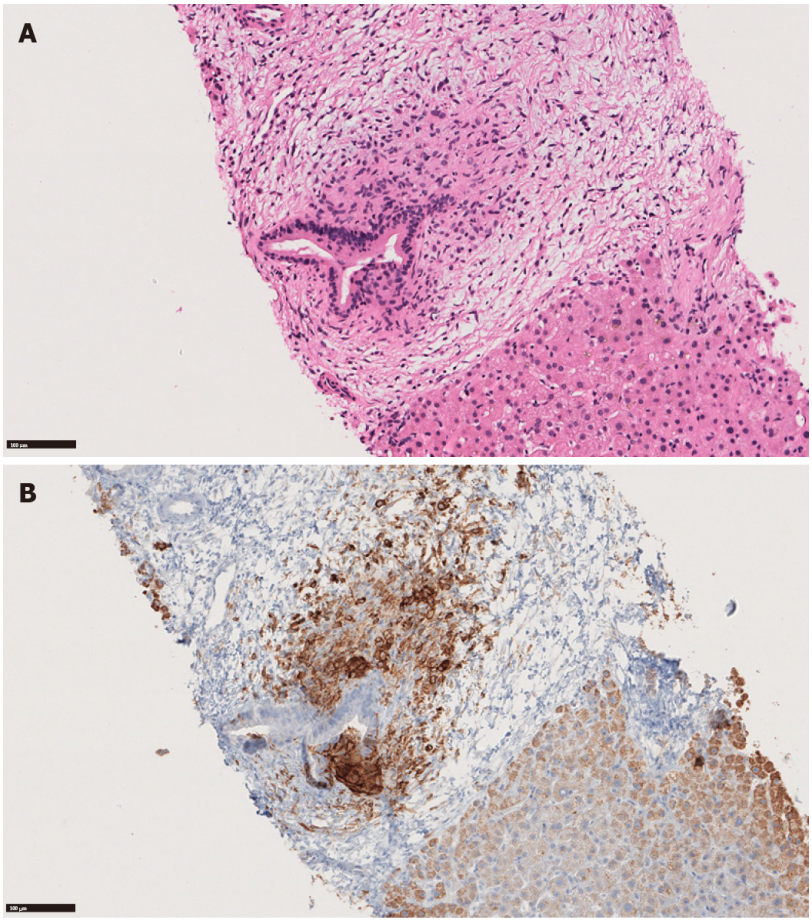


Figure 1 Hepatic Langerhans cell histiocytosis mimicking primary biliary cholangitis. A: Portal non-necrotizing granulomatous inflammation encasing a duct (Hematoxylin and eosin, 200 ×); B: CD1a immunostain highlights Langerhans cells surrounding the duct (CD1a, 200 ×). Image A was originally published in Cureus. Citation: Li H, Ells P, Arslan ME, Robstad KA, Lee H. Hepatic Langerhans Cell Histiocytosis (LCH) Presenting as a Harbinger of Multisystem LCH. *Cureus* 2020; 12: e8591 [PMID: 32676232 DOI: 10.7759/cureus.8591]. Copyright © The Authors 2020. Published by Cureus, Inc.[21].

efficacy in patients with *BRAF V600*-mutant LCH[31]. In addition, MEK inhibitors showed near-universal responses in patients with histiocytoses, including LCH, regardless of tumor genotype[32]. However, prospective clinical trials would be required to determine optimal duration of therapy and to explore a potential for combination with other targeted or cytotoxic therapies.

The optimal treatment for hepatic LCH remains to be determined. Currently, systemic chemotherapy is the mainstay of treatment. Yi *et al*[33] reported that earlier systemic chemotherapy has led to a relatively better outcome in hepatic LCH. However, the therapy was not effective once cirrhosis developed[33]. Therefore, early diagnosis and treatment of hepatic LCH would be crucial. Unfortunately, a subset of hepatic LCH patients develops severe sclerosing cholangitis that progresses to cirrhosis. In this case, liver transplantation may be the treatment of choice and should be considered early in the disease course[34]. Tang *et al*[35] reported a case of hepatic LCH that was successfully treated by liver transplantation with subsequent tacrolimus and mycophenolate mofetil as immunosuppressants[35].

Further collaborative studies regarding the biology, clinical presentation and outcome of hepatic LCH would be required to explore and refine variable treatment options.

PROGNOSIS

In LCH, liver involvement has a significant bearing on survival. The overall prognosis of hepatic LCH is poor with a fatality rate of 30%-50% (versus < 10% without liver involvement) and median survival of 9 years[6,18,20]. The 3-year survival rate of LCH with liver involvement is 51.8%, compared with that of 96.7% without liver involvement[6]. In Abdallah *et al*[20]'s study, 30% of patients died due to sclerosing

cholangitis complicated by secondary cirrhosis[20]. Therefore, it is very important to identify liver involvement at an early reversible stage. Early detection and treatment may improve the outcome[6,18].

CONCLUSION

While hepatic LCH is relatively uncommon, it portends a poor prognosis. Early detection and treatment of hepatic LCH may allow a better prognosis. Liver biopsy plays an important role in the diagnosis and management of hepatic LCH. Unfortunately, the histomorphology is non-specific and needs to be differentiated from other granulomatous processes, such as PBC or infection. A definitive diagnosis requires confirmatory immunohistochemical staining with CD1a and S100. Currently, systemic chemotherapy is the mainstay of treatment for hepatic LCH. Further studies are required to explore other treatment modalities including molecularly targeted therapy.

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ORIGINAL ARTICLE

Basic Study

Thymoquinone anticancer activity is enhanced when combined with royal jelly in human breast cancer

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Abstract

BACKGROUND

Breast cancer is the most common cause of the majority of cancer-related deaths in women, among which triple-negative breast cancer is the most aggressive type of breast cancer diagnosed with limited treatment options. Thymoquinone (TQ), the main bioactive constituent of *Nigella sativa*, has been extensively studied as a potent anticancer molecule against various types of cancers. Honeybee products such as the royal jelly (RJ), the nutritive secretion fed to honeybee queens, exhibit a variety of biological activities besides its anticancer effect. However, the anticancer activity of the combination of TQ and RJ against breast cancer is still unknown.

AIM

To investigate cytotoxicity of RJ in FHs 74 Int cells and the anticancer effects of TQ, RJ, and their combinations in the MDA-MB-231 cell line.

METHODS

Cells were treated with TQ, RJ, and their combinations for 24 h. Using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, we determined the half-maximal inhibitory concentration of TQ. Trypan blue and 3-(4,5 dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were then performed to assess the cell viability in response to different treatment conditions. Cell death and cycle regulation were investigated using propidium iodide deoxyribonucleic acid staining followed by flow cytometry in response to a single dose of TQ, RJ, and their combination. Immunostaining for cleaved caspase 3 and Ki67 expression

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was used to determine apoptosis induction and changes in cell proliferation.

RESULTS

TQ alone inhibited cell viability in a dose-dependent manner at concentrations below and above the half-maximal inhibitory concentration. RJ exhibited relatively nontoxic effects against MDA-MB-231 cells and FHs 74 Int small intestinal cells at concentrations below 5 μg/mL. High doses of RJ (200 μg/mL) had greater toxicity against MDA-MB-231 cells. Interestingly, the inhibition of cell viability was most pronounced in response to 15 μmol/L TQ and 5 μg/mL RJ. A dose of 15 μmol/L TQ caused a significant increase in the PreG1 population, while a more pronounced effect on cell viability inhibition and PreG1 increase was observed in response to TQ and RJ combinations. TQ was the main inducer of caspase 3-dependent apoptosis when applied alone and in combination with RJ. In contrast, no significant regulation of Ki67 expression was observed, indicating that the decrease in cell viability was due to apoptosis induction rather than to inhibition of cell proliferation.

CONCLUSION

This study is the first to report enhanced anticancer effects of TQ and RJ combination against MDA-MB-231 breast cancer cells, which could confer an advantage for cancer therapy.

Key Words: Anticancer activity; Breast cancer cells; Drug combination; Natural products; Royal jelly; Thymoquinone

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Core Tip: Royal jelly enhances thymoquinone (TQ) anticancer activity against breast cancer. TQ induces the apoptotic response in breast cancer cells while royal jelly when combined with TQ potentiates the reduction in cell viability more than each drug alone.

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INTRODUCTION

According to the global cancer project GLOBOCAN 2020, 19.3 million new cancer cases and 10 million cancer deaths have occurred in 2020[1]. The World Health Organization predicted that the global burden of cancer would double to about 29-37 million new cancer cases by 2040. Their report estimated the trend of future cancer cases by which breast cancer will account for 2778850 cases in 2040[2]. Breast cancer is the most common cancer among women worldwide[3] and is responsible for the majority of cancer-related deaths among women[4]. Invasive ductal carcinoma (IDC) is the most frequent type of breast tumor, followed by invasive lobular carcinoma (ILC). Together they make up 90% of breast cancers, while the remaining 10% is caused by particular types of none-ILC/none-IDC tumors[5-7]. Triple-negative breast cancer is an aggressive type of breast cancer with limited treatment options[8,9].

The human breast cancer cell line MDA-MB-231, a highly invasive and poorly differentiated triple-negative breast cancer (TNBC), was employed in our study. Being one of the most commonly used breast cancer cell lines in medical research, MDA-MB-231 derives from pleural effusion in metastatic mammary adenocarcinomas[10]. The absence of estrogen receptor, progesterone receptor, and human epidermal receptor 2 expression renders them nonresponsive to hormonal treatments[11]. However, these cells possess high invasive capacity and metastatic potential as they degrade the extracellular matrix of tissues and metastasize into lung, bone, or brain-specific cancers [12-14]. Conventional treatment of breast cancer comprises surgical procedures,

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radiotherapy, chemotherapy, endocrine (hormonal) therapy, and targeted and immunotherapies[15]. Despite the therapeutic impact of conventional treatments, they exert numerous side effects. Thus, extensive research has been conducted on alternative treatments utilizing plant-derived natural products with relatively non-toxic effects and high therapeutic potential.

Thymoquinone (TQ), the main bioactive constituent of *Nigella sativa* L. (Ranunculaceae), modulates the hallmarks of cancer[16] in addition to its cytoprotective[17,18], immunomodulatory, anti-oxidant, and anti-inflammatory activities[19,20]. Previous studies reported that TQ alone and in combination with natural and chemical agents act to inhibit breast cancer[21-23]. For instance, TQ in combination with piperine, lowered vascular endothelial growth factor expression, enhanced serum interferon-γ levels and apoptosis induction, and shifted the immune response toward T helper1 responses against EMT6 epithelial breast cancer[24]. TQ was shown to induce apoptosis when used alone or combined with amoxifen and suppressed the growth, viability, and invasion of breast cancer cell lines[25,26] through the regulation of the Akt signaling pathway[27].

Royal jelly (RJ), the nutritive secretion secreted from the mandibular and hypopharyngeal glands of worker bees *Apis mellifera* (Hymenoptera, Apidae), is the only food of the queen bee at larval and adult life and is responsible for fertility and prolonged life span[28,29]. Similar to TQ, RJ exerts various biological activities, including wound healing[30], anti-oxidant[26,31], immunomodulatory, and anti-inflammatory[32] activities, and anti-hypercholesterolemic[33,34], anti-hypertensive[35], anti-aging[36,37], and anticancer activities[38,39]. RJ was found to inhibit the proliferation of estradiol-induced cell proliferation of MCF-7 breast cancer cells[38] and reduce the volume of the 4T1 breast mammary tumor[39]. RJ also inhibited the proliferation of human colorectal adenocarcinoma cells[40], neuroblastoma[41], and vascular endothelial growth factor-induced migration, proliferation, and tube formation in human umbilical vein endothelial cells[42].

The poor prognosis of TNBC and its ability to resist chemotherapy and meta-stasize [43,44] made combination therapy a necessary option. TQ was successfully combined with several agents to enhance its anticancer therapeutic efficacy; however, TQ anticancer activity was not tested in combination with RJ. In a previous study, we showed that TQ exerted a dose-dependent antitumor effect against a panel of human colon cancer cell lines with minimal cytotoxicity against FHs 74 Int non-tumorigenic human intestinal cells. Here, to assess the cytotoxic effects of RJ, FHs 74 Int intestinal cell line was used as a model of non-tumorigenic epithelial cells. In our study, we investigated the anticancer activity of TQ and RJ combination *in vitro* against the MDA-MB-231 human TNBC cell line. Significant inhibitory effect of TQ and RJ combination was revealed by the enhanced cell death effects in MDA-MB-231 cell line. TQ was the main inducer of apoptosis mediating cell death mechanisms by inducing caspase 3 dependent apoptosis in a dose-dependent manner.

MATERIALS AND METHODS

Materials

MDA-MB-231 human breast cancer and FHs 74 Int human small intestinal cell lines were purchased from ATCC (Manassas, VA, United States). Dulbecco's Modified Eagle Medium (DMEM) and DMEM-F12 cell culture media were purchased from Lonza (Verviers, Belgium). TQ, trypsin-ethylenediamine tetraacetic acid, Dulbecco's phosphate-buffered saline (PBS), horse serum, fetal bovine serum (FBS), penicillin-streptomycin, dimethyl sulfoxide (DMSO), 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), trypan blue and methanol were purchased from Sigma-Aldrich (St. Louis, MO, United States). Insulin used for FHs 74 Int cell line culture (Actrapid 100 IU/mL) was purchased from the pharmacy at the American University of Beirut Medical Center. 4′,6-diamidino-2-phenylindole stain was purchased from Abcam (Cambridge, United Kingdom). Rabbit caspase 3 polyclonal antibody (9662) was purchased from Cell Signaling Technology (Danvers, MA, United States). Goat anti-rabbit polyclonal secondary antibody, Alexa Fluor 568 (A11011) was purchased from Invitrogen, Thermo Fisher Scientific (Carlsbad, CA, United States). Rabbit Ki67 monoclonal primary antibody (Cell Marque 275R-15) and donkey anti-rabbit Cy3 secondary antibody (Jackson 711-165-152) were provided by Dr. Noel Ghanem, Professor of Biology, American University of Beirut. Crude RJ was purchased from the bee farm at Rashaya al-Wadi, Lebanon, located at 1200-1600 m above sea level. A variety of seasonal plants predominate at this altitude and contribute to the diet of

bees, among which are Brassicaceae (Nasturtium), Anacardiaceae (Rhus), Ulmaceae (Ulmus), Rosaceae (Rosa), and Apiaceae (Eryngium). RJ was collected during the summer season of 2018-19 and stored at -20 °C.

Cell culture conditions

MDA-MB-231 was used as the model of an aggressive breast cancer cell line, while FHs 74 Int cell line was used as the model of a non-tumorigenic epithelial cell line. Both cell lines were cultured in their respective media in two-dimensional monolayer conditions. MDA-MB-231 cells were grown in DMEM cell culture media supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin (penicillin-streptomycin with penicillin at 10000 units and streptomycin at 10 mg/mL), and 10 μg/mL insulin was added to grow FHs 74 Int cells. All cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

Dissolution of RJ

Fresh RJ was supplied to our lab in the form of a solid extract. Knowing that RJ contains both polar and non-polar compounds, we considered mixing the crude RJ with DMSO, a polar aprotic solvent that is miscible with dH_2O and that is capable of dissolving hydrophilic and hydrophobic compounds[45,46]. Different proportions of dH_2O to DMSO were used to determine the ratio that produces the best solubility. Complete solubility was only obtained upon dissolving 20 mg of RJ in a solution of 800 μL dH_2O and 200 μL DMSO solution at 37 °C for 30 min along with vortexing every 10 min. Therefore, this protocol was used to prepare fresh RJ stocks prior to every treatment. Fresh RJ stock was then used to prepare dilutions needed for experiments.

Drug preparation and treatment

Directly before use, fresh stock of the purified synthetic compound TQ of 0.1 mol/L concentration was prepared by dissolving 16.4 mg of TQ crystals in 1 mL methanol. TQ stock was then diluted in respective media to obtain different TQ concentrations ranging from 1 μmol/L to 100 μmol/L used in cell treatment. RJ fresh stock was prepared by dissolving 20 mg in 200 μL DMSO mixed with 800 μL distilled water solution at 37 °C for 30 min. Intermediate concentrations of RJ ranging between 0.01 and 200 μg/mL were then prepared by serial dilutions from stock and used in cell treatment. In all experiments, treatment with TQ and RJ each alone or in combination was performed at 50% cell confluency. Treatment with TQ-RJ combination was done by adding TQ and RJ, each alone in wells containing their respective media and incubating cells with this mixture at different concentrations for 24 h.

MTT cell viability assay

All cells were seeded in 96-well plates at a density of 10000 cells/well, then treated for 24 h. Cell viability was then assessed by MTT that measures the ability of metabolically active cells to convert tetrazolium salt into violet formazan crystals. Cells were incubated with 120 μL of MTT solution (5 mg/mL prepared in 1 × PBS) for 3 h at 37 °C. Afterward, the solution containing the MTT dye was removed and replaced with 100 μL isopropanol to dissolve the formazan crystal. MTT optical density was then measured using a microplate reader enzyme-linked immuno-sorbent assay at 595 nm. Cellular viability was expressed as a percentage of metabolically active cells in treated conditions relative to control. Cell viability was reported as an average of three independent experiments, each condition in sextuplicate. RJ inhibitory effect was not determined by MTT due to its interference with the colorimetric absorbance measures of the MTT assay (data not shown).

Trypan blue exclusion assay

FHs 74 Int cells were seeded in 24-well-plates at a density of 70000 cells/well, while MDA-MB-231 were seeded in 12-well plates at a density of 20000 cells/well. Following treatment of cells for 24 h, alive and dead cells were collected. Samples were centrifuged at 1300 rpm for 5 min. Then, pellets were resuspended in DMEM growth medium, and trypan blue was added to the cell suspension in a 1:1 ratio. Next, cells were counted using a hemocytometer under the Axiovert inverted microscope at 10 × magnification. Cells stained blue were counted as dead, and results are expressed as a percentage of total cells. Cell viability was reported as an average of three independent experiments, each condition in duplicates.

Combination index analysis

The interaction between TQ and RJ was assessed using the Chou-Talalay plot[47]. Combination indices (CI) were calculated from the mean affected fraction at each drug combination using CompuSyn software (CompuSyn, Inc. Paramus, NJ, United States). CI > 1, CI = 1, and CI < 1 indicate antagonistic, additive, and synergistic effects, respectively.

Cell cycle analysis

MDA-MD-231 cells were seeded in 6-well plates at a density of 80000 cells/well. Cells were treated with 0.1 μg/mL RJ and 15 μmol/L TQ each alone. After 24 h, cells were collected and washed twice with 1 × PBS, fixed in 70% ice-cold ethanol, and stored at −20 °C for at least 1 d. Subsequently, cells were washed twice with 1 × PBS and incubated for 30 min at 37 °C with 100 μL of propidium iodide (PI) solution [6 μL RNase, 30 μL PI (1 mg/mL)]. Supernatants were then transferred to flow tubes with 200 μL PBS added. Cell cycle analysis was performed using the Fluorescence-activated cell sorting scan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, United States) and the Cell Quest software (Becton-Dickinson) was used to analyze the distribution of cells in the different phases of the cell cycle.

Immunofluorescence assay

MDA-MB-231 cells were plated on coverslips in 12-well plates at a density of 60000 cells/well. The medium was then removed, and the cells were treated with either TQ, RJ, or combinations. After treatment, the cells were washed twice with 1 × PBS and fixed at room temperature for 20 min in 4% formaldehyde. The formaldehyde was then removed, and the cells were washed three times in PBS before permeabilization in 0.5% Triton solution for 5 min. After two successive washes in PBS, cells were blocked in blocking buffer with FBS for 1 h at room temperature. Apoptosis was assessed using the caspase 3 antibody, which was subsequently diluted (1:500) in 3% bovine serum albumin and incubated separately with the cells overnight at 4 °C. The primary antibody was removed the next day, and the cells were washed three times in PBS supplemented with 0.1% Tween 20 before incubation for 1 h with goat anti-rabbit secondary antibody diluted (1:200) in 3% bovine serum albumin at room temperature. Finally, the secondary antibody was removed, and the cells were washed three times in PBS with 0.1% Tween 20 before staining the nuclei with 4′,6-diamidino-2-phenylindole and mounting on a glass slide. To evaluate cell proliferation, the same immunostaining protocol was followed for Ki67 immunofluorescence with minor modifications, including the preparation of Ki67 primary antibody and donkey anti-rabbit Cy3 secondary antibody solutions in blocking buffer with donkey serum at dilution 1:500 and 1:200 ratios, respectively. Also, cells were washed three times in PBS only after the removal of the primary and secondary antibodies. Imaging and visualization were performed using the microscope Zeiss Axio. For cleaved caspase 3 and Ki67 biomarkers, an equal number of representative images were taken for each slide in all conditions. The percentage of apoptotic cells expressing cleaved caspase 3 was then calculated while Ki67 immunofluorescence intensity was measured by ZEN lite Digital Imaging Software.

Statistical analysis

Unless otherwise stated, data are presented as mean ± standard error of the mean of three independent experiments with statistical analysis performed by one way analysis of variance (non-parametric) multiple comparison test on Graph Pad Prism V.7. Software (La Jolla, CA, United States). Statistical significance was set with a 95% confidence interval at $P < 0.05$.

Confocal Imaging

Cells were visualized and imaged by Axiovert inverted microscope from Zeiss at 10 × magnification. Confocal images were taken on Confocal Microscope Zeiss LSM710 at 40 × oil immersion magnification.

RESULTS

Cytotoxicity of TQ and RJ on human breast cancer cells

In a previous study, we showed that TQ doses up to 60 μmol/L exert minimal cytotoxic effects on normal intestinal cells[48]. Post 24 h treatment, TQ at concen-

trations below 15 μmol/L did not exert any statistically significant toxicity on MDA-MB-231 cell line relative to the control. Cell viability decreased remarkably, reaching 47% at 20 μmol/L, revealing that the half-maximal inhibitory concentration (IC_{50}) value was around 20 μmol/L (Figure 1A). The decline in cell viability was more pronounced with increasing TQ concentrations, indicating that TQ exhibited significant anticancer activity against MDA-MB-231 human breast cancer cells in a dose dependent manner. RJ exerted mild inhibitory effects at low doses of RJ (below 5 μg/mL) on FHs 74 Int non-tumorigenic human intestinal cells and MDA-MB-231 human breast cancer cells. However, at higher doses ranging from 10 μg/mL to 200 μg/mL of RJ, a more pronounced decrease in cell viability was observed, suggesting that very high doses of RJ are toxic to FHs 74 Int non-tumorigenic cells with a greater toxicity being exerted on breast cancer cells (Figure 1B and C). The IC_{50} of RJ was estimated to be 216 μg/mL in MDA-MB-231 cells, while it was much higher (292 μg/mL) in the FHs 74 Int cell line. Based on these results, relatively non-toxic doses of RJ ranging between 0.1-5.0 μg/mL were combined with TQ in subsequent experiments.

Anticancer effect of TQ, RJ, and their combinations against breast cancer

Next, we determined the effects of the combination of increasing doses of both RJ and TQ on cell viability. As shown in Figure 2A and B, no significant reduction in MDA-MB-231 viability was detected upon the treatment with RJ alone at doses of 0.1 μg/mL and 5 μg/mL or with TQ alone at doses below 10 μmol/L. Treatment with 5 μg/mL RJ, when combined with 5 μmol/L or 7.5 μmol/L of TQ caused 21% and 29% inhibition in cell viability, respectively (Figure 2B). The anti-tumor effects were more pronounced upon treatment with higher TQ doses. A dose of 10 μmol/L TQ in combination with 0.1 μg/mL RJ or 5 μg/mL RJ yielded a significant decrease in MDA-MB-231 cell viability by 40% and 58%, respectively. Treatment with 5 μg/mL RJ in combination with 10 μmol/L or 15 μmol/L of TQ decreased cell viability by 58% and 74%, respectively. These findings confirm the more potent anti-tumor effects upon combination treatment with higher RJ doses compared to each drug alone. The inhibition of cell viability (58% inhibition) by this combination treatment was greater than the sum of inhibition observed by each compound alone (6% and 12% inhibition by TQ and RJ alone, respectively), suggesting a synergistic effect. CIs were then calculated using CompuSyn software, confirming the synergistic interaction between both compounds in all the combinations tested with a CI value < 1. Anti-tumor effect was most pronounced (CI = 0.584) upon the combination of 5 μg/mL RJ with 10 μmol/L TQ (Figure 2C). Therefore, RJ enhances TQ anti-tumor activity against breast cancer by inducing dose dependent cell death effects.

TQ alone and in combination with RJ increases PreG1 population in breast cancer cells

To confirm further cell death and determine whether the inhibition of cell viability by RJ and TQ treatment of MDA-MB-231 cells was associated with changes in cell cycle regulation, cell cycle analysis using PI deoxyribonucleic acid staining with flow cytometry was performed. Cell death was significantly enhanced in response to TQ alone and when TQ was combined with 0.1 μg/mL RJ, a relatively non-cytotoxic dose (Figure 3A and B). In comparison with the control, the PreG1 population increased significantly upon the treatment with 15 μmol/L TQ alone, while a greater elevation was obtained when this dose of TQ was combined with 0.1 μg/mL RJ (Figure 3B). The increase in the PreG1 population was associated with a notable reduction in the G0/G1 and G2/M populations.

TQ and RJ combinations induce apoptotic cell death in breast cancer cells

To identify the mechanism of action responsible for the enhanced cell death effect of TQ and RJ combination treatment, we assessed the apoptotic effects of each compound alone and their combinations in MDA-MB-231 cell line. Insignificant increase in apoptosis levels was reported upon treatment with 0.1 μg/mL or 5 μg/mL of RJ. Confocal micrographs showed enhancement of apoptosis in response to the TQ and combination treatment, as evidenced by the increase in apoptotic nuclear bodies in MDA-MB-231 cells (Figure 4A). A significant increase in active caspase 3 expression was observed in response to treatment with 10 μmol/L and 15 μmol/L TQ alone, yielding a respective increase of 52% and 73% of caspase 3 expression in MDA-MB-231 cells (Figure 4B). Similar results were obtained upon treatment with 0.1 μg/mL RJ in combination with 10 μmol/L and 15 μmol/L TQ, while a more pronounced apoptotic effect was observed in response to treatment with 5 μg/mL RJ in combination with 10

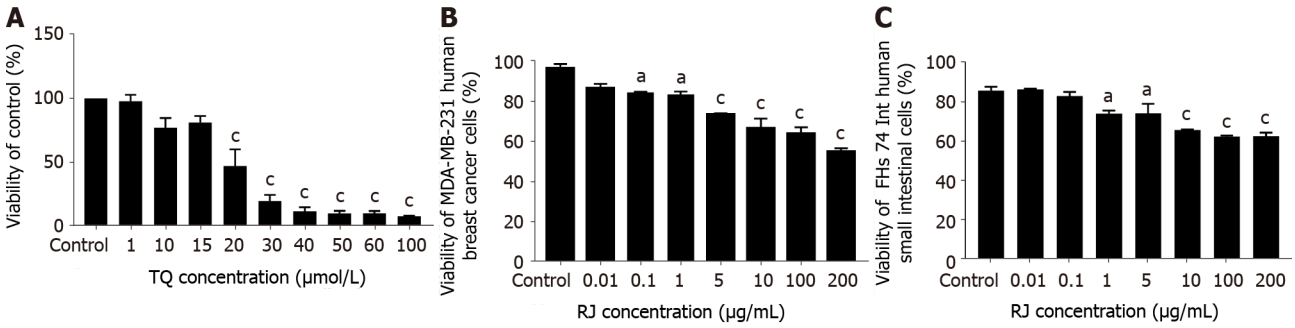
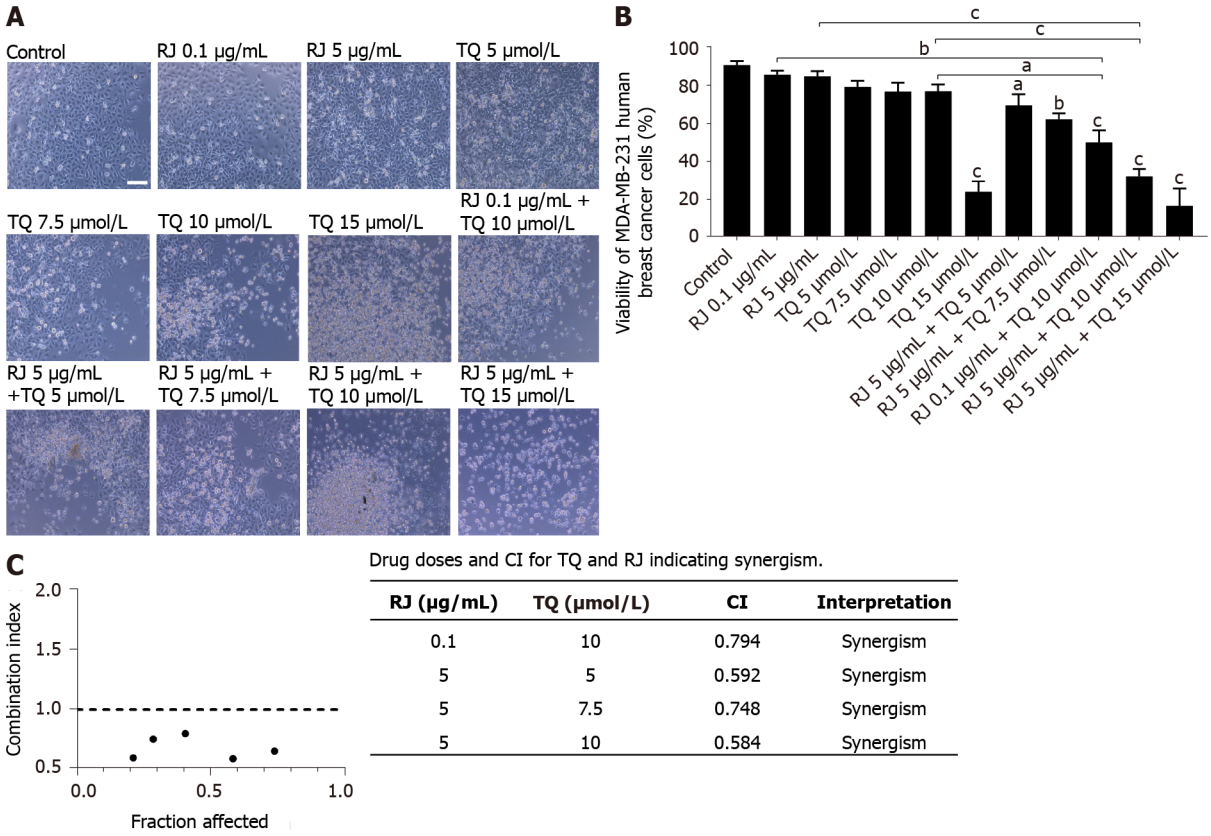


Figure 1 The inhibitory effect of thymoquinone and royal jelly on the viability of MDA-MB-231 and FHs74 Int cell line. A: 3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showing the percentage viability of MDA-MB-231 cell line and the half-maximal inhibitory concentration of thymoquinone (TQ) on MDA-MB-231 human breast cancer cell line after 24 h of treatment with different TQ concentrations. Cell viability was estimated by measuring the absorbance of the cell suspension after incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; B: Trypan blue exclusion assay showing the percentage cell viability after 24 h of treatment with different royal jelly concentrations on FHs74 Int; C: MDA-MB-231 cell lines. Data shown are an average of 3 independent experiments for panels A and B, and 2 independent experiments for panel C, respectively, expressed as mean ± standard error of the mean. Asterisks represent statistically significant results compared to the control, ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$). RJ: Royal jelly; TQ: Thymoquinone.



Drug doses and CI for TQ and RJ indicating synergism.

RJ (μg/mL)	TQ (μmol/L)	CI	Interpretation
0.1	10	0.794	Synergism
5	5	0.592	Synergism
5	7.5	0.748	Synergism
5	10	0.584	Synergism

Figure 2 Royal jelly and thymoquinone combinations enhanced the inhibition of MDA-MB-231 human breast cancer cell viability. A: Representative light microscopy images of MDA-MB-231 viability in response to different treatments. Cells were visualized by Axiovert inverted microscope from Zeiss at 10 × magnification with scale bar = 10 μmol/L; B: Trypan blue exclusion assay showing the percentage viability of MDA-MB-231 cell line after 24 h of treatment with different concentrations of royal jelly (RJ), thymoquinone, and combinations. Data shown are an average of three independent experiments expressed as mean ± standard error of the mean. Asterisks represent statistically significant results compared to the control and treatment conditions, ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$); C: Fraction affected-combination index plot showing combination index (CI) values plotted as a function of fraction affected values corresponding to the % cell death of five different combinations of thymoquinone (5 μmol/L, 7.5 μmol/L, 10 μmol/L and 15 μmol/L) and royal jelly (0.1 and 5 μg/mL) in MDA-MB-231 cells. The dotted line is the reference line, where CI value is equal to 1; circles in black represent CI values at different Fa. CI > 1, CI = 1, and CI < 1 indicate antagonistic, additive, and synergistic effects, respectively. CI: Combination index; RJ: Royal jelly; TQ: Thymoquinone.

μmol/L and 15 μmol/L TQ. Cell proliferation was then evaluated after 24 h of treatment by measuring the intensity of Ki67 fluorescence, a sensitive and specific proliferation biomarker. A minimal non-significant decrease in Ki67 expression was observed in response to all doses of RJ and TQ combinations as compared to the control (data not shown). This effect was confirmed by confocal imaging showing the

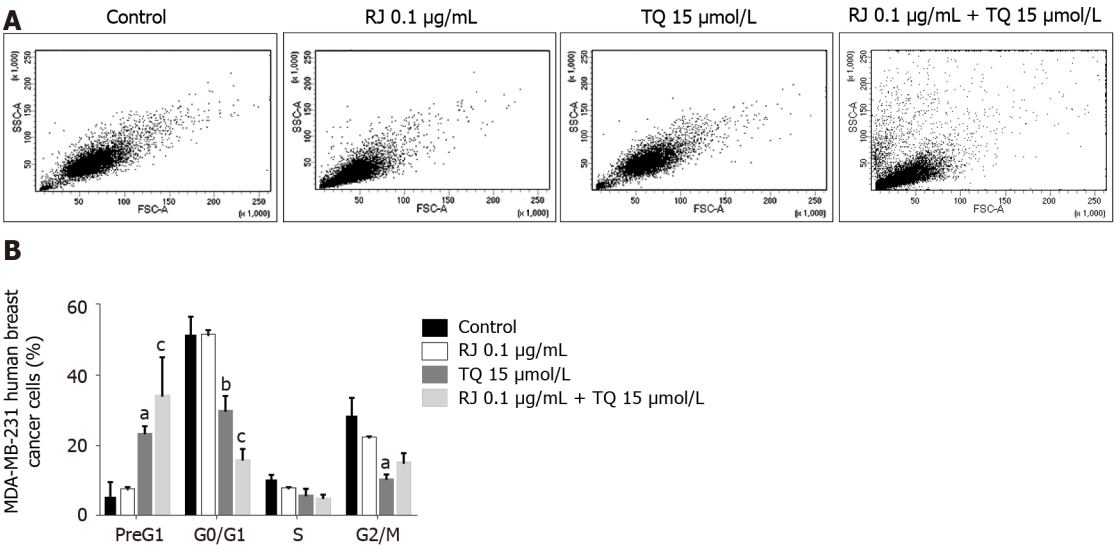


Figure 3 Cell death is enhanced by thymoquinone alone and by the combination thymoquinone and royal jelly. A: Representative density plots showing MDA-MB-231 cell distribution as a function of side scatter area and forward scatter area in the control and post-treatment with 15 µmol/L thymoquinone (TQ) and 0.1 µg/mL royal jelly alone and in combination for 24 h; B: Propidium iodide staining with flow cytometry showing the increase in Pre G1 upon treatment with TQ alone and the combination of TQ and royal jelly. Data shown are an average of three independent experiments expressed as mean ± standard error of the mean and analyzed by a two-way analysis of variance test followed by multiple comparisons test. Asterisks represent statistically significant results compared to the control, ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$). RJ: Royal jelly; TQ: Thymoquinone.

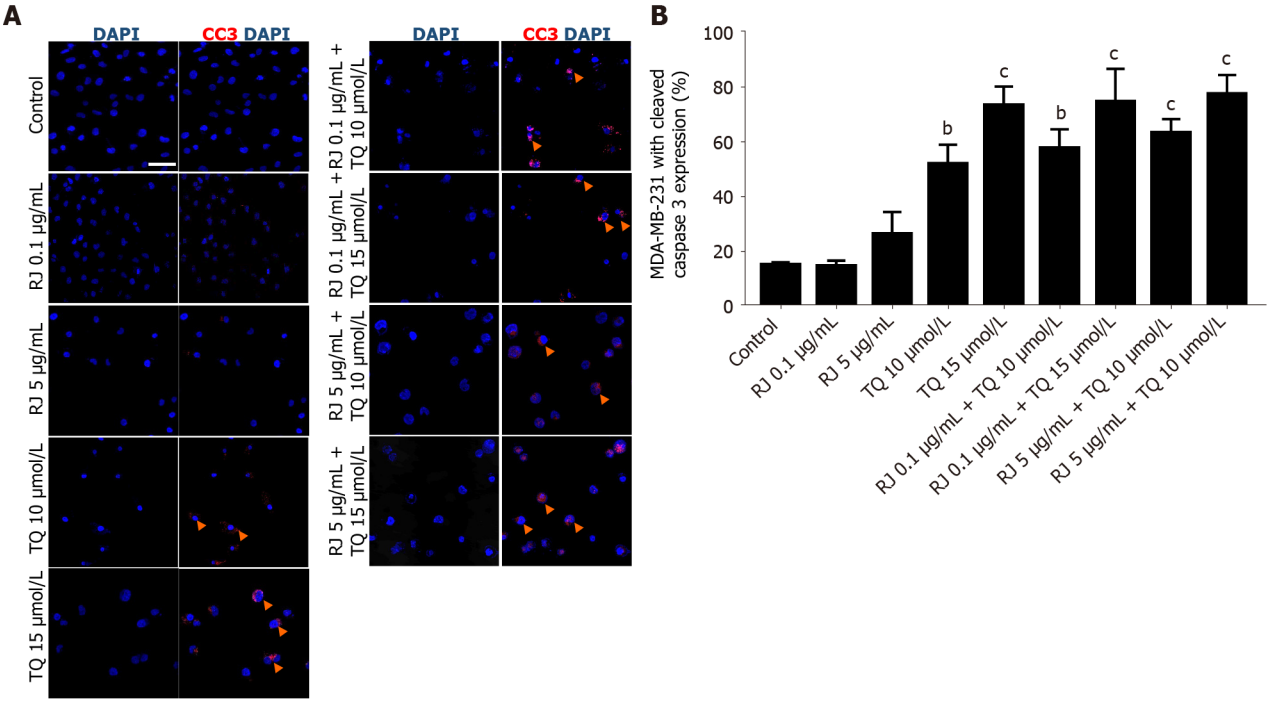


Figure 4 Effect of royal jelly, thymoquinone and combinations on caspase 3 cleavage in MDA-MB-231 human breast cancer cells. A: Immunofluorescence micrographs of cleaved caspase 3 expression at 24 h after treatment. Red indicates cleaved caspase 3 expression and blue indicates nuclei counter stained by 4',6-diamidino-2-phenylindole. Arrows indicate apoptotic nuclei. Nuclei were visualized by confocal Zeiss Axio microscope, 40 × oil immersion with scale bar = 50 µmol/L; B: Quantification of cleaved caspase 3 in MDA-MB-231 cells at 24 h of treatment with different concentrations of royal jelly, thymoquinone, and their combinations. Data shown are an average of 3 independent experiments expressed as mean ± standard error of the mean. Asterisks represent statistically significant results, ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$). RJ: Royal jelly; TQ: Thymoquinone.

modest change in the nuclear expression of Ki67 in response to the different treatments in MDA-MB-231 cells (data not shown). Therefore, RJ alone and TQ alone or their combinations did not modulate the expression of Ki67 in MDA-MB-231 human TNBC.

DISCUSSION

Breast cancer, the most common cancer among women, is identified as a heterogeneous disease arising from the differential expression of hormonal receptors along with genomic and intratumoral heterogeneity[5]. Despite the tremendous improvement in the therapeutic approaches, conventional treatments of breast cancer, including systemic therapy, exert organ-specific toxicity along with various side effects [49]. The interest in alternative treatments relying on relatively non-toxic and cost-effective natural resources has surged over the past decades, particularly from medicinal plants and honeybee products. TQ, the main constituent of *Nigella sativa* essential oil, and the nutritious honeybee secretions of RJ were shown to have potent anticancer activities against many types of cancers, including breast cancer[50,51].

Our study is the first to investigate the anticancer activity of both TQ and RJ alone and in combination against the triple-negative MDA-MB-231 human metastatic breast cancer cell line. TQ has been shown to possess potent anticancer activities against various cancer types, including colon cancer, with minimal cytotoxic effects on normal intestinal cells[48]. In the context of breast cancer, previous studies reported TQ-mediated induction of apoptosis, growth inhibition, in addition to suppression of viability and invasion of MDA-MB-231 and MCF7 cell lines mainly through the inhibition of Akt phosphorylation[23,25]. In line with these studies, we further confirmed TQ's anticancer activity in MDA-MB-231 cell line as evidenced by the dose-dependent cell death effects at concentrations below and above the IC_{50} value of 20 μmol/L. Our results showed that RJ exhibited minimal toxicity on FHs74 Int cell line at doses below 5 μg/mL, while a more pronounced inhibitory effect was observed at higher doses with a clear saturation effect obtained at doses equal or greater than 100 μg/mL. This indicates that RJ is relatively nontoxic to the non-tumorigenic human small intestinal cell line at doses equal or below 5 μg/mL. In line with the previously published studies[38,39], our findings demonstrate that RJ inhibits the viability of breast cancer cells. RJ exerted low to mild dose-dependent inhibitory effects on the viability of MDA-MB-231cell line at doses below 5 μg/mL. Cell death was more pronounced in MDA-MB-231 at 200 μg/mL RJ, suggesting the greater toxicity of RJ to breast cancer cells with the IC_{50} estimated to be 1.4 fold greater in FHs 74 Int cell line compared to that of MDA-MB-231 cell line.

Combination therapy is usually used to enhance the therapeutic response and overcome any possible drug resistance in cancer patients[52]. To assess for any possible anticancer synergy (or additive effects), concentrations that are not highly cytotoxic to cells (*i.e.* less than 50% cell death) should be used. Therefore, the anticancer effect of TQ in combination with RJ was assessed using drug doses below the IC_{50} values (*i.e.* 15 μmol/L). We documented an enhanced anticancer activity of TQ when combined with RJ against MDA-MB-231 cell line. Cancer cell viability decreased significantly in response to different combinations as compared to the treatment with each drug alone. Cell death was amplified by 3- and 5-fold in response to the combination of 5 μg/mL of RJ with 10 μmol/L and 15 μmol/L of TQ, respectively, with the lowest combination index obtained upon the combination of 5 μg/mL RJ with 10 μmol/L TQ, suggesting synergistic interaction. These results are consistent with the previous studies that reported the synergism of TQ in combination with different agents including melatonin[53] and piperine[24] against breast cancer, diosgenin on squamous cell carcinoma[54], docetaxel against prostate cancer[55], in addition to arsenic and interferon-alpha against human T-cell leukemia/lymphoma[56].

Cell cycle analysis using propidium iodide staining was performed to confirm further cell death and to examine whether TQ and RJ alone or in combination affect the cell cycle progression of breast cancer cells. In accordance with our findings using trypan blue exclusion assay, RJ alone at a dose of 0.1 μg/mL did not exert significant changes in cell viability compared to the control. Consistent with previous studies reporting the inhibitory activity of TQ[48,57,58], our study reports 4-fold increase in the PreG1 cell population, which was associated with a significant decrease in G0/G1 and G2/M cell populations in response to 15 μmol/L of TQ, further confirming TQ-mediated cell death. Interestingly, combining 15 μmol/L of TQ with 0.1 μg/mL RJ yielded a more pronounced cell death effect evidenced by the 6-fold increase in the PreG1 population. These results indicate that the cell death effect is enhanced upon the combination of TQ with RJ compared to single treatments with each compound alone. This indicates that RJ enhances the cell death effects of TQ in metastatic breast cancer.

To understand the underlying mechanism of the observed reduction in the viability of metastatic breast cancer cells upon the different treatments, we investigated apoptosis induction as a possible mechanism of cell death. Enhanced induction of apoptosis was evidenced by the increase in caspase 3 cleavage in response to the

increasing TQ doses alone or in combination with RJ. On the other hand, treatment with RJ alone did not induce any significant apoptotic effect compared to the control. Apoptotic cell death was increased by 4-fold in response to the combination of 10 μmol/L of TQ with 0.1 μg/mL and 5 μg/mL RJ, while a 5-fold increase was obtained upon combining 15 μmol/L of TQ with both RJ doses. Our results indicate that TQ is the main inducer of apoptosis, although an augmented apoptotic response was observed upon the combination with RJ, indicating that RJ could modestly potentiate the anticancer activity of TQ in TNBC. Our findings are consistent with previous studies showing induction of apoptosis upon treatment with TQ alone and in combination with other agents against cancer cells[24,56,59,60]. In line with previously published data[59], our study shows minimal changes in Ki67 intensity in response to TQ. Therefore, cell death of MDA-MB-231 cells was not due to the inhibition of proliferation but rather to apoptosis induction, as evidenced by the enhanced caspase 3 cleavage.

CONCLUSION

In conclusion, RJ and TQ, each being relatively non-toxic to normal cells, exhibit enhanced anti-tumor activities against human metastatic breast cancer when combined. Although TQ and RJ combination enhances apoptotic cell death, TQ appears to act as the main inducer of apoptosis mediating cell death by inducing caspase 3 dependent apoptosis in a dose-dependent manner. The combination of these two natural compounds deserves further investigation to identify the key molecules responsible for this enhanced anticancer activity.

ARTICLE HIGHLIGHTS

Research background

Despite the tremendous improvement in therapeutic approaches, triple-negative breast cancer has poor prognosis. Thymoquinone (TQ), the main constituent of *Nigella sativa* seeds and royal jelly (RJ), the honeybee secretion fed to honeybee queens, are effective against cancer. However, the anticancer activity of the combination of TQ and RJ against aggressive human breast cancer cells is yet unknown.

Research motivation

To establish novel treatments for breast cancer using natural, relatively non-toxic compounds with significant therapeutic value. We focused on investigating the anticancer activity of TQ and RJ combinations against triple-negative breast cancer.

Research objectives

This study aimed to characterize the anticancer activity of TQ and RJ alone and their combination *in vitro* against human triple-negative breast cancer.

Research methods

The inhibitory effect of TQ on triple-negative breast cancer cells was assessed by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Trypan blue exclusion assay was used to evaluate cell viability in response to different treatment conditions. Propidium iodide deoxyribonucleic acid staining followed by flow cytometry was performed to evaluate possible cell cycle regulation and cell death effects. Apoptosis and cell proliferation were determined using immunofluorescence assays for cleaved caspase 3 and Ki67 expression, respectively. The interaction between TQ and RJ and combination indices were evaluated using CompuSyn software.

Research results

TQ inhibited MDA-MB-231 breast cancer cell viability in a dose-dependent manner. RJ at low doses was relatively nontoxic to non-tumorigenic FHs 74 Int small intestinal epithelial cells, while at high doses greater toxicity against MDA-MB-231 breast cancer cells was observed. Inhibition of cell viability and cell death effects were more pronounced in response to TQ and RJ combinations compared to each drug alone. The reduction in breast cancer cell viability was mainly due to TQ-mediated caspase 3-dependent apoptosis.

Research conclusions

RJ and TQ are relatively non-toxic to normal cells and exhibited pronounced anticancer effects against human metastatic breast cancer. Although our findings demonstrate the potent pro-apoptotic activity of TQ compared to that of RJ, this is the first report of a significant enhancement in TQ's anticancer activity when combined with RJ.

Research perspectives

The reduction in breast cancer cell viability and enhanced cell death effects upon TQ and RJ combinations highlights their potential therapy for human triple-negative breast cancer.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic role of sarcopenia in metastatic colorectal cancer patients during first-line chemotherapy: A retrospective study

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Abstract

BACKGROUND

Sarcopenia is a condition characterized by decreased skeletal muscle mass due to physiological ageing or to a concomitant disease such as neoplasia. In cancer patients, a low lean body mass is suggested to be a negative prognostic factor for survival and for the development of dose-limiting chemotherapy toxicities irrespective of disease stage.

AIM

To evaluate the prognostic role of sarcopenia in patients with metastatic colorectal cancer (mCRC) undergoing first-line chemotherapy.

METHODS

Our retrospective analysis included 56 mCRC patients who received first-line chemotherapy from 2014 to 2017 at the Medical Oncology Unit of our hospital. Computerized scans were performed before starting chemotherapy and at the first disease reassessment. Sarcopenia was assessed using the skeletal mass index = muscle area in cm^2/(height in m^2) calculated at the L3 vertebra. Overall survival and objective response rate were evaluated. Toxicities were analyzed during the first four cycles of therapy and graded according to Common Terminology Criteria for Adverse Events version 4.0. A loss of skeletal muscle mass ≥ 5% was

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considered indicative of deterioration in muscle condition.

RESULTS

Median age was 67 years and 35.7% of patients were ≥ 70 years old. Fourteen patients (25%) were sarcopenic at baseline computed tomography (CT) scan (7/33 men; 7/23 women); 5/14 sarcopenic patients were ≥ 70 years old. Median follow-up was 26.8 mo (3.8-66.8 mo) and median overall survival was 27.2 mo (95%CI: 23.3-37.3). Sarcopenia was not correlated to overall survival (P = 0.362), to higher toxicities reported during the first 4 cycles of chemotherapy (P = 1.0) or to response to treatment (P = 0.221). At the first disease reassessment, a skeletal muscle loss (SML) ≥ 5% was found in 17 patients (30.3%) 3 of whom were already sarcopenic at baseline CT scan, while 7 patients became sarcopenic. SML was not correlated to overall survival (P = 0.961). No statistically significant correlation was found between baseline sarcopenia and age (P = 1.0), body mass index (P = 0.728), stage at diagnosis (P = 0.355) or neutrophil/lymphocyte ratio (P = 0.751).

CONCLUSION

Neither baseline sarcopenia nor SML affected survival. In addition, baseline sarcopenia was not related to worse treatment toxicity. However, these results must be interpreted with caution due to the limited sample size.

Key Words: Sarcopenia; Lean body mass; Skeletal muscle mass; Metastatic colorectal cancer

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Core Tip: According to previous studies, sarcopenia is associated with a poorer prognosis in metastatic colorectal cancer (mCRC) patients. We analyzed the prognostic role of sarcopenia in 56 mCRC patients treated with first-line chemotherapy. Neither sarcopenia nor muscle mass loss was significantly associated with survival. Other prospective studies are needed to clarify the role of sarcopenia in mCRC patients. Moreover, greater efforts should be made to diagnose sarcopenia earlier to correct strength and muscle mass, and thus improve patient tolerability to treatment and survival.

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INTRODUCTION

In medical oncology, the dose of cytotoxic drugs is calculated according to the patient's body surface area (BSA) using formulae devised in the early twentieth century and validated on a limited number of subjects[1,2]. For instance, the formula proposed by Du Bois *et al*[2], which is routinely used in adult cancer patients, was based on data of only nine patients. Furthermore, individualized dosage of antineoplastic agents based on BSA does not necessarily equate to a patient's drug exposure because the quantity of active drug circulating in the body and the duration of circulation may vary due to various factors[3,4]. In fact, pharmacokinetic parameters of a particular agent, such as area under the curve and clearance, may differ substantially among patients not only because of genetic factors, pharmacological interactions and the physiological characteristics of patients, but also because of body composition variations that are typical of the natural history of cancer patients[1,5].

The total body mass consists of two major compartments, fat and lean, which are the major sites of distribution of lipophilic and non-lipophilic drugs, respectively[6]. Therefore, the ratio of fat and lean tissue masses could be a better parameter than BSA

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with which to determine the dose of cytotoxic agents, as it affects metabolism, plasma concentration and the toxicity of chemotherapy drugs[6,7]. Moreover, patients with a similar or identical body weight, BSA or body mass index (BMI) may have a different lean body mass (LBM)[8,9]. Skeletal muscle tissue accounts for most of the LBM and is the predominant source of proteins which are essential for all cell processes[4,10]. People with a low skeletal muscle mass may have a lower volume of drug distribution and reduced protein binding compared to people with a normal muscle mass thereby resulting in a higher plasma drug concentration and worse treatment toxicity[8,11]. The skeletal muscle mass decrease due to physiological ageing or concomitant disease such as neoplasia is defined as "sarcopenia"[12]. In cancer patients, a low LBM and sarcopenia are negative prognostic factors for survival[8,9,13] and for the development of dose-limiting chemotherapy toxicities[6,14] irrespective of disease stage. The aim of the present study was to retrospectively analyze the prevalence of sarcopenia in patients with metastatic colorectal cancer (mCRC) and its prognostic role.

In 2018, the European Working Group on Sarcopenia in Older People 2 published an updated definition that uses low muscle strength as the primary parameter for recognizing sarcopenia, together with additional items of low muscle quantity or quality[15]. However, due to the retrospective nature of our analysis, we used the computed tomography (CT) scans performed at the time of first diagnosis of metastatic disease to evaluate the muscle area, and therefore the muscle quantity, at the level of the third lumbar vertebra.

MATERIALS AND METHODS

The primary end-point of this study was to assess the association between baseline sarcopenia, estimated before starting first-line chemotherapy, and overall survival (OS) in mCRC patients. The secondary end-points were: (1) to evaluate the potential correlation of baseline sarcopenia with the objective response rate (ORR) to first-line chemotherapy and with the development of side effects to antineoplastic therapy during the first four cycles of treatment; (2) to investigate the association between skeletal muscle loss (SML) at first disease reassessment and OS; and (3) to examine the relationship between sarcopenia and age, BMI, disease stage at the time of first diagnosis and the neutrophil/lymphocyte ratio (NLR) as an inflammation index.

Our retrospective analysis included 56 mCRC patients who received first-line chemotherapy for metastatic disease from 2014 to 2017 at the Medical Oncology Unit of the Federico II University Hospital. All patients had signed the informed consent document for the use of personal data in the medical record according to the Italian privacy legislation. The study was approved by the panel of scientists proposing the research and by all the collaborators who participated in the research and it was conducted in accordance with the 2013 Declaration of Helsinki.

Computerized scans were performed before starting chemotherapy (baseline) and at first disease reassessment (2-3 mo after starting therapy). The images were analyzed by a subspecialty trained abdominal radiologist. Sarcopenia was assessed using the skeletal mass index [SMI = muscle area in cm^2/(height in m^2)][16]. The cross-sectional area of all skeletal muscles was calculated at the third lumbar vertebra on pre-contrast axial CT images with a slice thickness of 5 mm, using the open-source Horos software (version 3.3.6)[16,17]. An attenuation threshold ranging from -29 to 150 Hounsfield units was set for muscle tissue[16].

Sarcopenia was defined by Martin SMI cut-offs[8], that combined both sex-specific and BMI cut-offs: 43 cm^2/m^2 for men with BMI < 25 kg/m^2, 53 cm^2/m^2 for men with BMI ≥ 25 kg/m^2 and 41 cm^2/m^2 for women regardless of BMI. A loss of skeletal muscle mass ≥ 5% from baseline CT to first disease reassessment was considered indicative of a deterioration in muscle condition[18]. Patients' characteristics were categorized as follows: age (< 70 years *vs* ≥ 70 years), BMI (underweight < 18.5 kg/m^2; normal weight 18.5-24.9 kg/m^2; overweight 25-30 kg/m^2 and obese > 30 kg/m^2) and disease stage at the time of first diagnosis (limited *vs* metastatic). A NLR ≥ 3 was considered as an inflammation index[19]. Toxicities were analyzed during the first four cycles of therapy and graded according to the Common Terminology Criteria for Adverse Events version 4.0. Survival was calculated from the date of baseline CT, at the time of metastatic disease diagnosis, to death or until the last outpatient visit. Disease status was assessed using the Response Evaluation Criteria in Solid Tumors version 1.1.

The statistical review of the study was performed by a biomedical statistician. Univariate and multivariate analysis and the calculation of the hazard ratio (95%CI) were carried out according to the Cox regression. Survival curves were estimated

using the Kaplan–Meier method. The Chi-squared test was used to correlate sarcopenia and ORR, toxicities, age, BMI, disease stage at the time of first diagnosis and NLR. The statistical analysis was performed using the SPSS version 20.0 software (SPSS Inc.).

RESULTS

We examined 56 consecutive mCRC patients who had received first-line chemotherapy and whose CT-scans were available in our archive. Fourteen patients (25%) were sarcopenic at baseline CT scan (7/33 men; 7/23 women). The median age of patients was 67 years (37-85 years) and 20 of the 56 patients (35.7%) were 70 years or older. Five of the 14 sarcopenic patients were 70 years or older. BMI distribution was 0% underweight, 37.5% normal weight, 39.3% overweight and 23.2% obese. SMI varied within each BMI category: 6/21 normal weight patients, 6/22 overweight patients and 2/13 obese patients were sarcopenic at baseline CT scan. Eighteen patients (32.1%) had a NLR ≥ 3. At the time of first diagnosis, 23 patients (41.1%) had II or III stage disease according to the pTNM classification and they subsequently developed metastases; 33 patients (58.9%) received the diagnosis at the metastatic stage. Of the 14 sarcopenic patients at the time of first diagnosis of metastatic disease, 4 had metachronous metastases and 10 had synchronous metastases.

The median follow-up was 26.8 mo (3.8-66.8 mo) and the median OS was 27.2 mo (95%CI: 23.3-37.3) (Figure 1). Sarcopenia was not correlated to either OS (HR, 0.72 95%CI: 0.35-1.47, P = 0.362) (Figure 2) or higher toxicity during the first 4 cycles of chemotherapy (P = 1.0) (Table 1). Four of the 14 (28.6%) sarcopenic patients and 13 of the 42 (31%) non-sarcopenic patients had at least one reduction in drug dosage due to toxicity during the first four cycles of therapy (P = 1.0). Twenty-seven patients (48.2%) had a partial or complete response, the disease was stable in 24 patients (42.8%), and 5 patients (8.9%) had disease progression as best response to first-line treatment. Response rate was not correlated to baseline sarcopenia (P = 0.221) (Table 1).

At first disease reassessment, 17 patients had an SML ≥ 5% (30.3%); 3 of these patients were already sarcopenic at baseline CT scan, while 7 patients became sarcopenic. Of these 6 men and 1 woman, 4 were under the age of 70 years; at baseline, 3/7 patients were normal weight, 3/7 were overweight and 1/7 was obese. One normal weight patient became overweight, while one overweight patient became normal weight at first disease reassessment. The median OS of these 7 patients was 27.93 mo, similar to that of the entire study population. Muscle mass loss was not correlated to OS (P = 0.961) (Figure 3).

No statistically significant correlation was found between baseline sarcopenia and age (P = 1.0), BMI (P = 0.728), stage at diagnosis (P = 0.355) and NLR (P = 0.751) (Table 1).

DISCUSSION

Skeletal muscle decrease is generally associated with physiological ageing. The probable mechanism of sarcopenia is an imbalance in muscle protein turnover due to endocrine changes (*e.g.*, reduction of sex hormones and growth factors), age-related cell damage and mitochondrial dysfunction, oxidative stress, low-grade systemic inflammation, physical inactivity and malnutrition[20-23]. In cancer patients the loss of muscle mass can occur earlier than in healthy people due to the synergy between physiologic and tumor factors (*e.g.*, production of inflammatory cytokines that induces a catabolic state)[24]. Depending on histology and disease stage, the prevalence of sarcopenia varies greatly among patients affected by neoplasia; for example, sarcopenia has been diagnosed in 30%-65% of patients with pancreatic neoplasia[25,26]; in 15.9%-66.9% of women with breast cancer[27,28]; in 47.9%-89% of gastric cancer patients[29-31]; in 27.5% of patients with advanced hepatocellular carcinoma[32]; in 52.5%-54.5% of patients with metastatic renal cell carcinoma[33,34]; in 19.4%-39% of patients with colorectal cancer[35,36].

In our study population, the prevalence of sarcopenia was 25%: 14 of the 56 patients were sarcopenic at baseline CT and most of them (9/14, 64%) were under the age of 70 years, which indicates that it is not uncommon to find a low skeletal mass in young adults. In this context, it is notable that Miyamoto *et al*[37] found that young CRC patients (< 65 years) with sarcopenia had a significantly shorter OS than those without sarcopenia, while the prognostic role of sarcopenia was lost in patients above 65 years

Table 1 Correlation between sarcopenia, age, body mass index, stage at diagnosis, neutrophil/lymphocyte ratio, toxicity and response to treatment, *n* (%)

	Sarcopenia			*P* value
	Total	No	Yes	
Age				1
≥ 70 yr	20 (35.7)	15 (35.7)	5 (35.7)	
BMI (kg/m^2)				0.728
18.5-24.9	21 (37.5)	15 (35.7)	6 (42.9)	
25-30	22 (39.3)	16 (38.1)	6 (42.9)	
> 30	13 (23.2)	11 (26.2)	2 (14.3)	
Stage at diagnosis				0.355
TNM II/III3	23 (41.1)	19 (45.2)	4 (28.6)	
TNM IV	33 (58.9)	23 (54.8)	10 (71.4)	
NLR				0.751
≥ 3	18 (32.1)	13 (31)	5 (35.7)	
Toxicity during the first 4 chemotherapy cycles				
At least one dose reduction	17 (30.4)	13 (31)	4 (28.6)	1
Diarrhea G ≥ 2	10 (17.9)	8 (19)	2 (14.3)	1
Neutropenia G ≥ 3/4	11 (19.6)	8 (19)	3 (21.4)	1
Response to treatment				0.221
Partial/complete response	27 (48.2)	18 (42.9)	9 (64.3)	

BMI: Body mass index; NLR: Neutrophil/lymphocyte ratio.

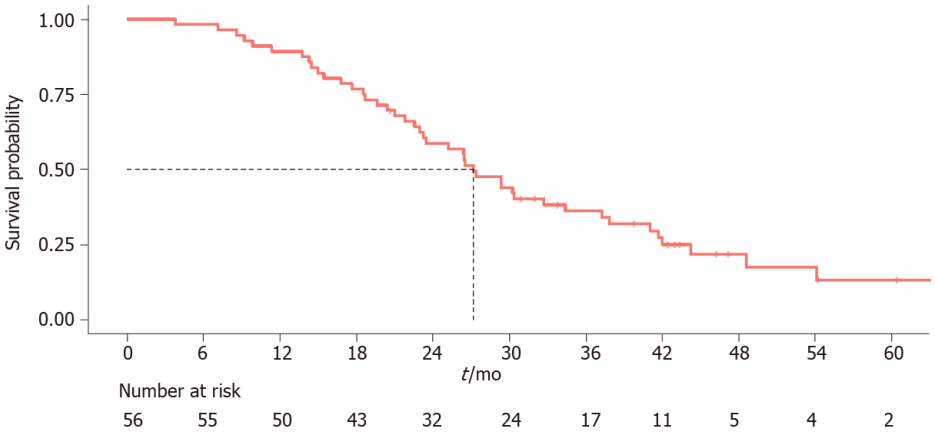


Figure 1 Overall survival.

of age. Consequently, it is also important to assess muscle mass in young CRC patients upon diagnosis, to better define the prognosis of each patient, and possibly to tailor anticancer treatment and improve the correction of sarcopenia. Indeed, various strategies have been reported to improve muscle mass and strength, namely exercise [38,39], dietary supplementation of proteins[40] and long-term intake of omega-3 fatty acids, which have anti-inflammatory and anabolic activities[41].

Clinicians should determine whether patients have sarcopenia not only regardless of age, but also regardless of BMI. In fact, a feature of sarcopenia that differentiates it from cachexia, is that it can occur without a concomitant loss of adipose tissue. In our study, no patient was underweight, and 8 of the 14 (57%) sarcopenic patients were overweight or obese (Figure 4). Notably, not all sarcopenic patients have a low BMI:

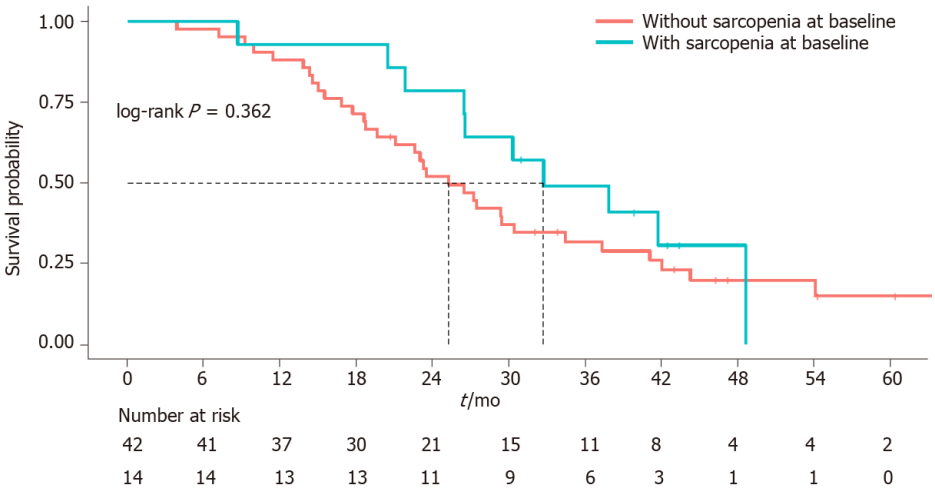


Figure 2 Overall survival according to baseline sarcopenia.

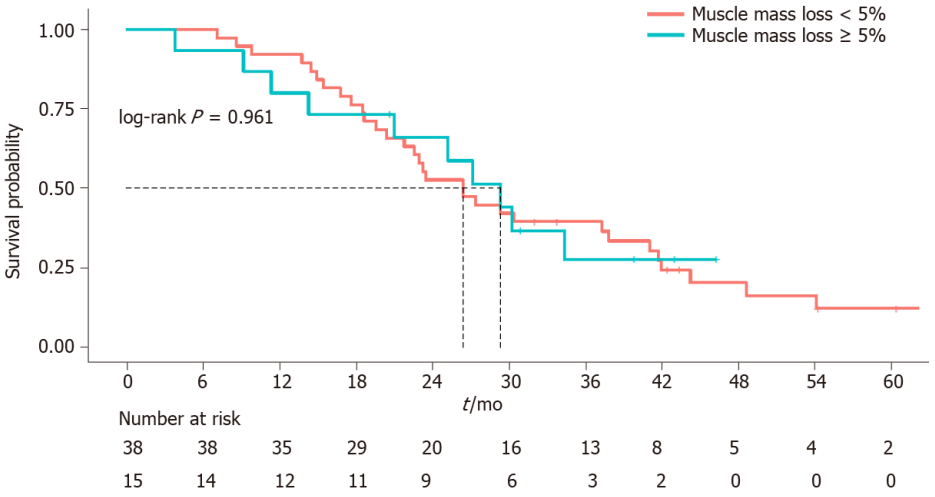


Figure 3 Overall survival according to muscle mass loss.

unlike other causes of muscle loss, sarcopenia can be associated with normal or even excessive body weight, *i.e.*, the so-called "sarcopenic obesity"[8,9]. The loss of muscle tissue can be associated with increased intramuscular fat, which results in a reduction in strength and muscle mass[40]. Martin *et al*[8] found that high weight loss, a low muscle index and low muscle attenuation due to fat infiltration, independently worsened survival in 1473 patients with lung or gastrointestinal cancer. Patients with all three of these poor prognostic variables survived 8.4 mo (95%CI: 6.5 to 10.3) regardless of BMI, in contrast to patients who had none of these features, who survived 28.4 mo (95%CI: 24.2 to 32.6; $P < 0.001$)[8]. In addition, BMI was predictive of survival, with the heaviest patients showing the longest survival. However, obese patients without any risk factorS survived 35.6 mo, which is twice longer than the median survival of the entire population (16.7 mo), while obese patients with three poor prognostic variables survived only 8.5 mo[8].

A low LBM and sarcopenia have been correlated to a worse prognosis and a worse quality of life in patients with solid tumors[6,7,13]. A meta-analysis of 38 studies, involving a total of 7843 patients, showed that subjects with a reduced SMI had a shorter OS, cancer-specific survival and disease-free survival than subjects with a normal SMI[13]. However, it included studies of various tumor types (*e.g.*, pancreaticobiliary cancer, hepatocellular carcinoma and esophageal cancer that have worse outcomes than other malignancies, such as colorectal cancer), disease stages (limited and advanced), therapeutic strategies, imaging techniques and sarcopenia cut-off values.

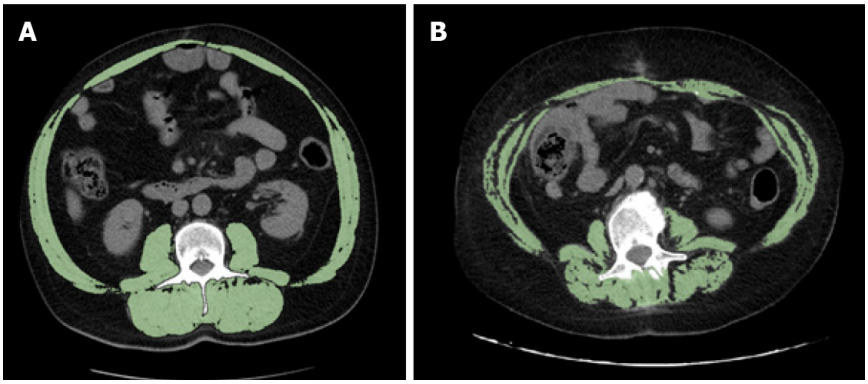


Figure 4 Representative examples of two obese patients with different skeletal mass index values. A: The patient is a 56-year-old man with body mass index (BMI) = 32.91 kg/m^2 and normal skeletal mass index (SMI) value = 62.70 cm^2/m^2; B: The patient is a 61-year-old woman showing BMI = 32.54 kg/m^2 and reduced SMI value = 39.45 cm^2/m^2.

In our patient cohort undergoing first-line chemotherapy for mCRC, sarcopenia was not related to either survival or response rate. Previous studies reported that low muscle mass was a negative prognostic factor both in resectable[37,42] and in advanced[43-45] colorectal cancer. However, those studies included patients with clinical and disease-related characteristics different to our patients. For example, Vashi *et al*[43] studied patients younger than ours (median age 53.3 years) who were at different disease stages (early and metastatic disease), some of whom had already been treated for metastatic disease. Moreover, they used cut-off values that did not consider gender or BMI in their definition of sarcopenia. Also the reports by Kurk *et al*[44] and Charette *et al*[45] were based on data derived from clinical trials designed for different endpoints, and included patients undergoing maintenance chemotherapy after the first therapeutic line or heavily pretreated patients. The latter two groups of patients have a better and worse prognosis, respectively, than our patients.

Differently, other studies did not find a correlation between basal sarcopenia and survival, but they suggested that muscle mass loss during treatment plays a negative prognostic role. For example, Miyamoto *et al*[18] analyzed 182 Asian patients with unresectable CRC. Female gender (P < 0.001) and BMI < 25 kg/m^2 (P < 0.001) were significantly associated with a lower SMI. There were no significant associations between baseline skeletal muscle mass, progression-free survival (PFS) and OS[18]. However, 22 patients with SML > 5% after first-line chemotherapy had significantly shorter PFS and OS *vs* those without SML (PFS, log-rank P = 0.029; OS, log-rank P = 0.009)[18]. Sasaki *et al*[46] found sarcopenia in 135 of 219 Asian mCRC patients (mostly male, older, with a lower BMI, lower visceral and subcutaneous fat content and a lower waist circumference than patients without sarcopenia). Baseline sarcopenia was not associated with prognosis, but SML ≥ 9% at 3 mo was associated with a high incidence of adverse events (P = 0.01), poor ORR (P < 0.01) and poor PFS (P = 0.03)[46]. Also Blauwhoff-Buskermolen *et al*[47] observed that the muscle area of 67 patients with mCRC (78% at first-line treatment and 22% at second-line treatment) decreased by 6.1% (95%CI: 28.4% to 23.8%; P < 0.001) during 3 mo of chemotherapy. Changes in muscle area were not associated with any treatment dosage modifications (dose reduction, delay or discontinuation), but patients with a muscle loss of 9% or more during treatment had significantly lower survival rates (at 6 mo, 33% *vs* 69% of patients alive; at 1 year, 17% *vs* 49% of patients alive; log-rank P = 0.001)[47].

We found no association between muscle loss during first-line treatment and survival, but it is interesting to note that a SML ≥ 5% occurred in 17 patients (30.3%); only 3 of whom were already sarcopenic at baseline CT scan, while 7 patients became sarcopenic during therapy. Chemotherapy probably induces progressive muscle damage both directly *via* a cytotoxic mechanism, and indirectly consequent to a more sedentary lifestyle because of the development of toxicity and asthenia.

In our analysis, sarcopenia was not related to higher toxicity reported during the first four cycles of chemotherapy, but 30.4% of all patients had at least one reduction in drug dosage due to toxicity, which indicates that approximately one-third of our patients did not receive an adequate drug dosage as calculated based on BSA. In this context, it is interesting to refer to the data reported by Prado *et al*[14], who examined 62 patients with stage II/III colorectal cancer receiving adjuvant treatment with 5-fluorouracil (5-FU). Exposure to 5-FU was then normalized per kilogram of LBM. In women, the 5-FU dose/kg LBM varied from 12.8 to 23 mg/kg LBM and, in men, from

12 to 20.1 mg/kg LBM[14]. Levels greater than or equal to 20 mg of 5-FU/kg of LBM were associated with an increased risk of developing dose-limiting toxicities (any grade 3/4 toxicity, dose delay or reduction) at first therapy cycle, especially in women [14]. The population analyzed in the latter study differed greatly from our patients as it included only early-stage colon cancer patients who had undergone surgery on the primary tumor and they were treated with a single adjuvant drug, administered with an obsolete schedule (5-FU and leucovorin by i.v. bolus for 5 d every 28 d); however, these data illustrate how drug exposure varies widely among patients and how this variation affects treatment tolerability.

Other studies investigated the correlation between low muscle mass and worse toxicity during chemotherapy. For instance, Ali *et al*[6] assessed data from one prospective (n = 80 patients) and one retrospective study (n = 58 patients) that included patients at different stages of CRC, treated with different therapeutic regimens with one or more drugs. They observed that a low LBM was an independent determinant of toxicity and neuropathy in patients administered a FOLFOX-based regimen (5-FU + oxaliplatin) using conventional BSA dosing[6]. Gökyer *et al*[48] evaluated 36 patients with mCRC who received regorafenib. Dose-limiting toxicity (DLT), defined as toxicity requiring dose reduction or drug withdrawal, occurred in 13 of the 23 patients (56.5%) with basal sarcopenia, whereas only 1 of the 13 patients (7.6%) without sarcopenia experienced DLT (P = 0.005)[48]. Kurk *et al*[44,49], using data of the randomized phase 3 CAIRO3 study[50], found that sarcopenia at the start of maintenance capecitabine + bevacizumab was not associated with DLT, whereas patients with > 2% SMI loss had a significantly higher risk of DLT. When capecitabine + oxaliplatin + bevacizumab was reintroduced due to disease progression, 25% of patients started the treatment at a reduced dose and most of them were patients with previous SMI loss[49]. Interestingly, after drug dose adjustment, no further DLT was observed in the subgroup of patients with SMI loss[49].

Currently, data on the prognostic and predictive role of sarcopenia are based mostly on retrospective studies or on clinical trials designed for other endpoints. Conflicting results highlight the need to investigate further the role of low muscle mass in cancer patients. Indeed, there is a need for prospective studies of more homogeneous populations in terms of age, sex, tumor histology, stage of disease, treatment setting, and mono- or polychemotherapy regimens. In the future, clinicians might evaluate the body composition of cancer patients before starting chemotherapy in order to select the drug (*e.g.* lipophilic, hydrophilic, immunotherapy or biological) with the shortest regime (for example, shortening induction therapy in favor of a weakened therapy in sarcopenic patients), the most adequate dosage, and ancillary support strategies (*e.g.* exercise, specific nutrition supplements, drugs, *etc.*).

CONCLUSION

In our study, neither baseline sarcopenia nor muscle mass loss during first-line chemotherapy influenced survival in mCRC patients. Moreover, baseline sarcopenia did not worsen treatment toxicities during first-line chemotherapy. However, these results must be interpreted with caution given the limited sample size. Further prospective studies are needed to investigate the actual role of sarcopenia in prognosis and therapeutic decision-making. Greater efforts should be made to diagnose sarcopenia upon cancer diagnosis to correct strength and muscle mass as early as possible and thus improve the patient's tolerability to treatment and survival.

ARTICLE HIGHLIGHTS

Research background

People with a low skeletal muscle mass, defined as "sarcopenia", may have a lower volume of drug distribution and reduced protein binding compared to people with a normal muscle mass thereby resulting in a higher plasma drug concentration and worse treatment toxicity. In cancer patients, sarcopenia is considered a negative prognostic factor for survival and for the development of dose-limiting chemotherapy toxicities.

Research motivation

Pharmacokinetic parameters of a given drug, such as area under the curve and

clearance, may differ substantially among patients depending on body composition. The ratio of fat to lean tissue mass could be a better tool than body surface area with which to determine the dose of cytotoxic agents as it affects metabolism, plasma concentration and the toxicity of drugs.

Research objectives

The primary end-point of this study was to assess the association between baseline sarcopenia, evaluated before starting first-line chemotherapy, and overall survival in metastatic colorectal cancer patients. The secondary end-points were to investigate: (1) the potential correlation of baseline sarcopenia with the objective response rate to first-line chemotherapy and with the development of side effects during the first four cycles of treatment; (2) the association between skeletal muscle loss (SML) at first disease reassessment and overall survival (OS); and (3) the relationship between sarcopenia and age, body mass index (BMI), disease stage at the time of first diagnosis and the neutrophil/lymphocyte ratio as an inflammation index.

Research methods

Computed tomography (CT)-scans were performed before starting chemotherapy and at the first disease reassessment. Sarcopenia was assessed using the skeletal mass index [SMI = muscle area in cm^2/(height in m^2)] calculated at the L3 vertebra. Sarcopenia was defined by Martin SMI cut-offs that combined both sex-specific and BMI cut-offs: 43 cm^2/m^2 for men with BMI < 25 kg/m^2, 53 cm^2/m^2 for men with BMI ≥ 25 kg/m^2, and 41 cm^2/m^2 for women regardless of BMI. OS and objective response rate were evaluated. Toxicities were analyzed during the first four cycles of therapy and graded according to Common Terminology Criteria for Adverse Events version 4.0. A loss of skeletal muscle mass ≥ 5% was considered indicative of deterioration in muscle condition.

Research results

The prevalence of sarcopenia was 25%: 14 of the 56 patients were sarcopenic at baseline CT and most of them (9/14, 64%) were under the age of 70 years, which indicates that it is not uncommon to find a low skeletal mass in young adults. No patient was underweight, and 8 of the 14 (57%) sarcopenic patients were overweight or obese. Sarcopenia was not correlated to overall survival (P = 0.362), to higher toxicities reported during the first 4 cycles of chemotherapy (P = 1) or to response to treatment (P = 0.221). At the first disease reassessment, a SML ≥ 5% was found in 17 patients (30.3%) 3 of whom were already sarcopenic at baseline CT scan, while 7 became sarcopenic. SML was not correlated to overall survival (P = 0.961).

Research conclusions

Although this is a negative study, our results must be interpreted with caution given the limited sample size. Moreover, the body composition of cancer patients should be evaluated before starting chemotherapy to better select the drug (*e.g.* lipophilic, hydrophilic, immunotherapy or biological) with the shortest regime (for example, shortening induction therapy in favor of a weakened therapy in sarcopenic patients), the most adequate dosage, and ancillary support strategies (*e.g.* exercise, specific nutrition supplements, drugs, *etc.*).

Research perspectives

There is a need for prospective studies of more homogeneous populations in terms of age, sex, tumor histology, stage of disease, treatment setting, and mono- or polychemotherapy regimens, to investigate the actual role of sarcopenia in prognosis and therapeutic decisions. Greater efforts should be made to diagnose sarcopenia upon cancer diagnosis in order to correct strength and muscle mass as early as possible and thus improve the patient's treatment tolerability and survival.

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SYSTEMATIC REVIEWS

Reduction of muscle contraction and pain in electroporation-based treatments: An overview

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Abstract

BACKGROUND

In the first studies of electrochemotherapy (ECT), small cutaneous metastases were treated and only mild or moderate pain was observed; therefore, pain was not considered a significant issue. As the procedure began to be applied to larger cutaneous metastases, pain was reported more frequently. For that reason, reduction of both muscle contractions and pain have been investigated over the years.

AIM

To present an overview of different protocols described in literature that aim to reduce muscle contractions and pain caused by the electroporation (EP) effect in both ECT and irreversible EP treatments.

METHODS

Thirty-three studies published between January 1999 and November 2020 were included. Different protocol designs and electrode geometries that reduce patient pain and the number of muscle contractions and their intensity were analysed.

RESULTS

The analysis showed that both high frequency and bipolar/biphasic pulses can be used to reduce pain and muscle contractions in patients who undergo EP treatments. Moreover, adequate electrode design can decrease EP-related morbidity. Particularly, needle length, diameter and configuration of the distance between the needles can be optimised so that the muscle volume crossed by the current is reduced as much as possible. Bipolar/biphasic pulses with an inadequate pulse length seem to have a less evident effect on the membrane permeability compared with the standard pulse protocol. For that reason, the number of pulses and the voltage amplitude, as well as the pulse duration and frequency, must be chosen so that the dose of delivered energy guarantees EP efficacy.

CONCLUSION

Pain reduction in EP-based treatments can be achieved by appropriately defining

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the protocol parameters and electrode design. Most results can be achieved with high frequency and/or bipolar/biphasic pulses. However, the efficacy of these alternative protocols remains a crucial point to be assessed further.

Key Words: Electrochemotherapy; Irreversible electroporation; Pain; Muscle contraction; Monopolar or monophasic pulses; Bipolar or biphasic pulses

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Core Tip: This is an overview of different published protocols that aim to reduce muscle contractions and pain due to the electroporation (EP) effect. The analysis showed that both high frequency and bipolar/biphasic pulses can be used to reduce pain and muscle contractions. Moreover, appropriate electrode design can lower EP-related morbidity.

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INTRODUCTION

Electrochemotherapy (ECT) is a locoregional anti-tumour therapy that combines a low dose of a chemotherapy drug with high-intensity electric pulses to induce cell membrane electroporation (EP). Consequently, the drugs enter the tumour cells and exert their cytotoxicity[1-4]. Unlike other antitumour treatments based on physical phenomena, ECT is able to exert a specific effect at the cellular level, causing the death of the treated tumour cells. Because it is highly effective in treating cutaneous and subcutaneous tumours regardless of histology[5-7], ECT treatment has been extended to more deeply located tumours[8-13].

To increase the efficacy of EP treatment, the voltage amplitude and the duration or the number of electric pulses are often increased, as long as the required current does not exceed the limit set by the pulse generator. The standard operating procedures[6] for ECT define the electric protocol that, combined with intra-tumour or intravenous delivery of bleomycin or cisplatin[14-19], guarantees an adequate efficacy of the therapy: A train of eight high voltage 100 μs monopolar electric pulses with a repetition frequency of either 1 Hz or 5 kHz is often used. However, the application of high voltage monopolar pulses may cause pain and muscle contractions[20]. For that reason, the use of muscle relaxants and general anaesthesia[21-23] are often required.

In the first studies on ECT, small cutaneous metastases were treated with the observation of only mild or moderate pain was[24-26]; therefore, pain was not considered a significant issue[6,7]. Subsequently, as the procedure started to be applied to larger cutaneous metastases, pain was reported more frequently[27,28]. For that reason, reduction of both muscle contractions and pain have been investigated over the years. The main improvements were achieved by applying pulses at a higher frequency[29-34] or by using special electrode designs.

Repetition frequency of electric pulses has a close relationship with muscle contraction, which leads to a painful burning sensation and patient complaints[19,20]. An increase in repetition frequency by reducing the pulse-to-pulse pause, seems to reduce unpleasant sensations that occur during ECT[24,35-42]. Moreover, many authors reported that electric pulses lasting microseconds at a high repetition frequency do not decrease ECT antitumour efficacy[35,38]. However, although the pulse frequency is related to muscle contraction, the pain sensation also depends on other pulse characteristics such as voltage amplitude and pulse number, duration and shape[36]. The electrodes used for ECT treatment can also affect the onset of pain. Particularly, needle length, diameter and configuration that changes the distance between needles can be optimised so that the muscle volume crossed by the current is reduced as much as possible. Electrodes with a smaller distance between needles are

less painful because they require lower voltages. However, they only treat small portions of tissue, and thus must be applied multiple times to cover the entire lesion.

More recently, it has been demonstrated that the use of high frequency irreversible electroporation (H-FIRE)[28-33], namely bursts of short high frequency bipolar pulses, can further reduce muscle contraction and the subsequent pain caused by the electric pulses. Treatment with H-FIRE pulses, however, may require an electric field intensity higher than the standard electric protocol, both for ECT and for irreversible electroporation (IRE), to reach an equivalent treatment efficacy. An additional disadvantage is delivering pulses at considerably higher voltage amplitudes[34].

The aim of this review is to present an overview of the different protocols proposed in the literature to reduce muscle contractions and pain caused by the EP effect in both ECT and IRE treatments. The main findings of a number of researchers are reported in the results section. The impact of different electrode designs is also considered, as the reduction of muscle contraction and patient morbidity can also be obtained with an appropriate electrode design.

MATERIALS AND METHODS

This review is the result of a self-study without protocol or a registration number. In order to ensure an adequate variety of the assessed studies, several electronic databases were searched: PubMed (United States National Library of Medicine, http://www.ncbi.nlm.nih.gov/PubMed); Scopus (Elsevier, http://www.scopus.com/); the Web of Science (Thomson Reuters, http://apps.webofknowledge.com/); and Google Scholar (https://scholar.goo-gle.it/). Only studies published between January 1999 and November 2020 were analysed because that time window is consistent with recent developments in the fields of ECT and IRE. Papers not indexed in the electronic databases were evaluated through the references of included studies. The systematic search for papers of interest is shown in the flow chart in Figure 1. The inclusion criteria evaluated the article title, abstract and contents and included pre-clinical and clinical studies that examined pain or muscle contractions caused by reversible or IRE treatments. Only articles written in English were included. Studies with insufficient reported data, case reports, reviews or letters to the editor were excluded Four investigators carried out data extraction from the included papers, focusing on the type of study (*i.e.* numerical analysis, *in vitro*, *in vivo* or *ex vivo*), the type of EP (ECT or IRE), the pulse characteristics and the main results regarding reduction of muscle contraction and pain.

RESULTS

A considerable number of protocols that aim at reducing muscle contractions and pain, caused by the EP effect were found in the literature. The research was conducted with the aim of identifying the parameters that are most responsible for the perception of pain and the stimulation of muscle contraction in patients undergoing EP treatment. Thirty-three studies published between January 1999 and November 2020 were retrieved and papers not indexed in the electronic databases were identified in their reference lists. As *per* the approach described in Figure 1, 15 studies did not meet the abstract inclusion criteria and were therefore rejected. Five papers were found to be case reports, reviews or editorial letters, did not satisfy the inclusion criteria and were not included in the analysis. The remaining thirteen articles[29,30,33,34,37-45] were included in this manuscript, as they met all the required criteria (Figure 2). Four papers described treatment of cutaneous and subcutaneous tumours[37,40,41,44], two papers included sarcomas[38,42] and pancreatic tumours[43,44] and six were conducted in healthy subjects or phantoms[29,30,33,34,39,45].

Reduction of muscle contraction and pain: ECT protocols

In a study published in 1999, Daskalov *et al*[36] compared monophasic and biphasic pulses *in vivo*. The monophasic pulse protocol consisted of eight exponentially or rectangular-shaped pulses of 100 μs with a frequency of 1 Hz. In the biphasic pulse protocol, a rectangular pulse of 50 + 50 μs without intra-pulse delay was used in two different ways: (1) Eight pulses with a 1 s interval; and (2) A single burst of eight pulses spaced at 1 ms, with a total duration of 7.1 ms. In both protocols, the selected pulse amplitude ranged from 750 V to 1250 V, depending on the tumour size, with a

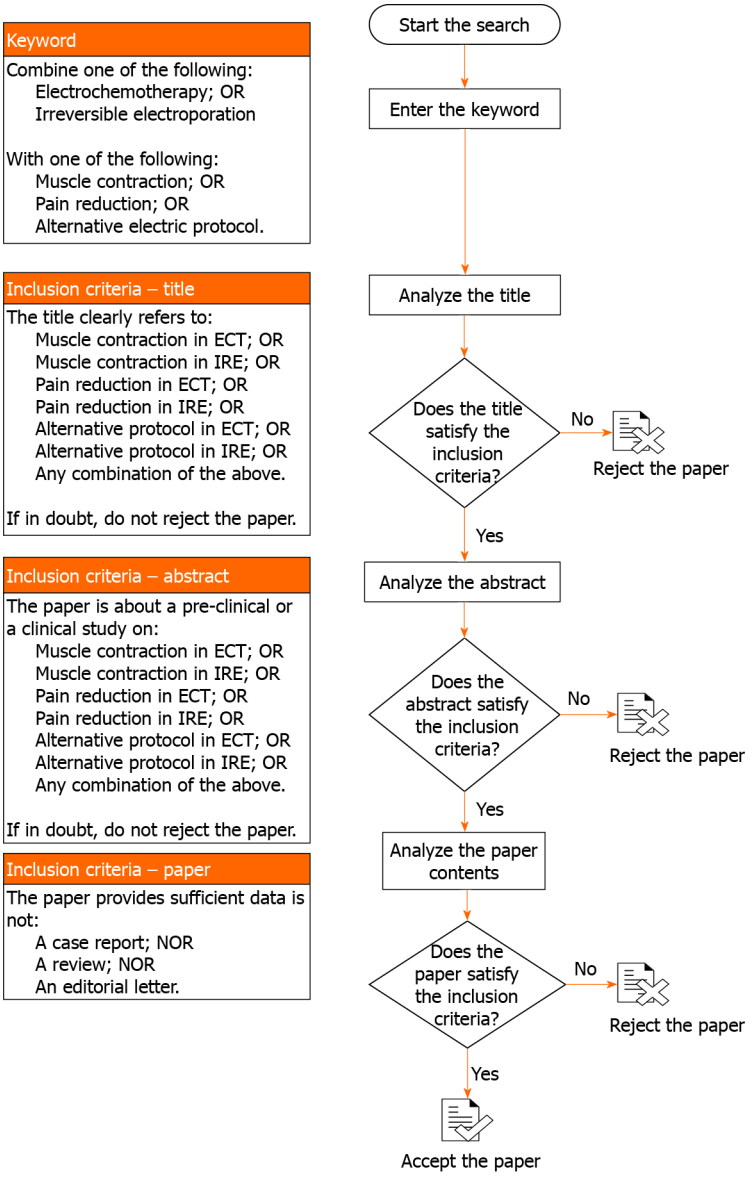


Figure 1 Systematic search of relevant literature. ECT: Electrochemotherapy; IRE: Irreversible electroporation.

resulting electrical field strength varying between 330 V/cm and 1250 V/cm. The study showed that the two pulse protocols (monophasic or biphasic) provided the same effect in terms of treatment result. However, the biphasic pulses were better tolerated by the patients. Particularly, the second biphasic mode, a single burst of eight pulses, was considered more acceptable than the first, which comprised eight separate stimuli. Thus, applying the pulses in a rapid sequence was as effective as the use of a larger inter-pulse interval and was better tolerated by the patients. The results of this study[36] were also confirmed by Melzack[46] who previously noted that increasing the number, (*N*) of applied pulses led to a better effect compared with increasing the pulse duration (*T*), provided that $N \times T$ was constant. In a later study, Miklavcic *et al* [37] demonstrated that pulse frequencies above the frequency of tetanic contraction (100 Hz) gradually reduced the number of individual muscle contractions. They identified muscle contractions associated with high voltage pulses as the main source of pain for patients undergoing ECT. When the pulse frequency was relatively low, the patient experienced separate muscle contractions associated with each delivered pulse. For that reason, the authors investigated the relationship between muscle contraction and pulse characteristics; particularly, repetition frequency and pulse amplitude. A train of eight 100 μs rectangular pulses at either low or high voltage amplitudes was used. At a low voltage (70 V), the measurements were performed at pulse repetition frequencies of 1, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 Hz. At a high voltage (250 V), the measurements were performed at 1, 100, 500, 1000 and 5000 Hz. To investigate the effect of both frequency and amplitude on muscle contraction, they measured the

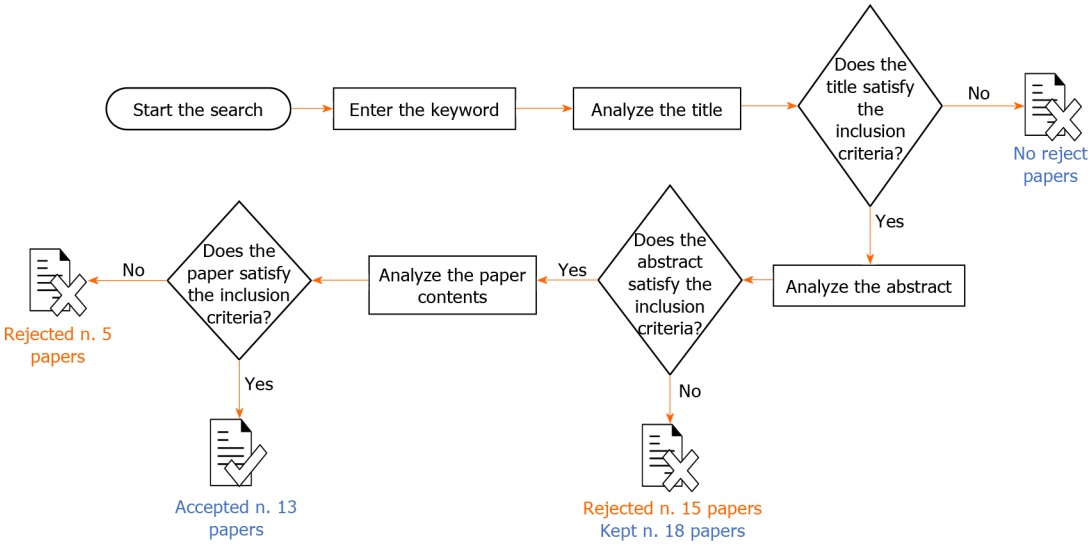


Figure 2 Included and excluded studies in the systematic review.

muscle torque in response to electric pulses. They also studied the antitumour efficacy of ECT at different pulse repetition frequencies to be sure that the pain reduction did not lead to a loss of treatment efficacy. Measurements of muscle torque confirmed that high frequency pulses reduced the number of individual contractions to a single muscle contraction. More precisely, with increasing pulse frequency, muscle torque increased up to the frequency of 100 or 200 Hz, reaching a maximum value of 0.16-0.24 nm; however, a further increase of frequency above 200 Hz reduced the muscle torque regardless of the pulse amplitude, with a mean value of about 0.07 nm at 5 kHz, a value similar to that observed during the application of 1 Hz pulse trains. Moreover, by increasing the frequency of electric pulses above the frequency of tetanic contraction (*i.e.* at pulse frequencies higher than 2000 Hz), the authors reported that, even if the muscle torque was similar to that observed in a typical ECT protocol (1 Hz pulse trains), the patients perceived only one muscle contraction instead of eight. Finally, *in vivo* experiments also demonstrated that ECT achieved similar efficacies regardless of the pulse frequency that was used (1 Hz-5 kHz), which suggest that there is a considerable potential for the clinical use of high frequency pulses in ECT.

The relationship of pulse frequency and muscle contraction and subsequent patient pain, was successively studied by Zupanic *et al*[38]. A train of eight electric pulses, of 1 Hz and 5 kHz repetition frequencies, was delivered to 40 healthy patients. After the conclusion of each protocol, the subjects completed the short-form McGill Pain Questionnaire[47] with separate visual analogue scales for pain intensity and unpleasantness. Their results confirmed what Miklavcic *et al*[37] had previously demonstrated, by finding that muscle contractions, which contribute to the discomfort felt by the subjects during the delivery of electric pulses, are strictly related to pulse frequency. When evaluating the sensorial and affective quality of pain (in the short-form McGill Pain Questionnaire, the most frequently selected pain descriptors were stabbing (80%), cramping (57.5%), throbbing (60%), shooting (60%) and hot-burning (53.8%). However, while both protocols of EP received similar average intensity scores for most descriptors (1.4 for stabbing, 1.0 for cramping, 1.1 for throbbing, 1.1-0.9 for shooting and 1.0-0.7 for hot-burning), treatment with 5 kHz electric pulses was less unpleasant. Therefore, the latter (P = 0.017) was preferred over the standard 1 Hz pulses, even though the perceived pain intensity, ranging from 6 mm to 94 mm, with similar visual analogic scores, was almost the same regardless of the frequency.

In a 2014 study, Spugnini *et al*[39] analysed the effects of biphasic pulse length on both treatment efficacy and morbidity. The authors investigated two different protocols of trains of eight biphasic pulses, at a voltage of 1300 V/cm. The standard protocol consisted of pulses lasting 50 + 50 ms each, with a frequency of 1 Hz and with 1 ms intra- and inter-pulse intervals. The investigational protocol consisted of pulses lasting 50 + 50 μs each, with a frequency of 1 Hz and with 10 μs intra- and inter-pulse intervals. The protocols were tested both *in vitro* (human lung cancer cell line A549) and *in vivo* (mice xenografts; privately owned rabbits with spontaneous tumours). Three of the mice treated with the standard protocol had a strong (grade 4) muscular contraction and the other four had a moderate (grade 3) muscular contraction. Mice

treated with the investigational protocol had muscular contractions reported as flicker (grade 1, two mice), weak (grade 2, four mice) and moderate (one mouse). The rabbits treated with the investigational protocol had muscular contractions graded as flicker (two rabbits), weak (three rabbits) and moderate (one rabbit). Given the results obtained from the study, it was concluded that the investigational protocol substantially reduced the morbidity associated with the delivery of electric pulses and achieved a significantly higher efficacy compared with the standard protocol.

In a more recent *in vitro* study in mouse skin melanoma (B16-F1) cells, Scuderi *et al* [40] delivered the electric pulses after adding 1–330 μM cisplatin. Two pulse protocols were evaluated: (1) Eight 100 μs monopolar pulses, 0.4–1.2 kV/cm, 1 Hz (standard ECT protocol); and (2) Eight bursts at 1 Hz, consisting of 50 bipolar pulses with 1 + 1 μs width, 0.5–5 kV/cm, 1 μs intra-pulse delay [high frequency EP (HF-EP)]. The analysis of the results was conducted by evaluating the difference between the two protocols (monopolar or bipolar pulses), focusing on their effect on both the efficacy of the treatment and the associated cytotoxicity. First, the results showed that both monopolar and bipolar pulse protocols, in combination with cisplatin, achieved the desired efficacy in killing cells. However, as the onset of membrane permeabilisation was higher in the HF-EP (2 kV/cm) protocol than in the ECT (0.8 kV/cm, $P = 0.036$) protocol, the bipolar pulse protocol needed a higher electric field (2 kV/cm, $P < 0.001$ *vs* 1.2 kV/cm, $P < 0.001$). Second, the results obtained suggest that HF-EP could be used in ECT with potential alleviation of muscle contractions and pain. In fact, even if the pain was not evaluated, it has been previously demonstrated that a short negative pulse delivered after a positive pulse accelerates passive repolarisation that abolishes the action potential. That means that fewer muscle contractions, and thus less pain, can be expected with HF-EP than with the classic 100 μs pulses. As the authors themselves concluded, although it is still at the *in vitro* testing stage, the clinical use of HF-EP pulses for ECT could potentially decrease the discomfort associated with muscle contractions and pain, and simplify the treatment procedure by lowering the dose of muscle relaxants and anaesthesia and avoiding synchronisation with the electrocardiogram.

Finally, in 2020, García-Sánchez *et al*[41] assessed the ability of sine waves to perform ECT. They compared the classic ECT protocol (eight squared unipolar pulses of 100 μs and 1 Hz repetition frequency, electric field of 1300 V/cm) with both bipolar square pulses and sinusoidal bursts. The analysed protocols (bipolar and sinusoidal) were made for pulses with no intra- nor inter-pulse pauses. The bursts were delivered at various frequencies between 10 and 100 kHz and with electric fields of at least 1300 V/cm, and the duration and number of pulses varied depending on the experiment. The authors also carried out a computer simulation to calculate the electric field distribution and the temperature increase during the delivery of the treatment. Furthermore, verification of the effectiveness of the treatment was essential in the comparison between the different protocols, which was taken into account by considering the tumour response. Specifically, the efficacy of the treatment was assessed by comparing sinusoidal bursts at three frequencies (10, 50 and 100 kHz) and two electric field intensities (1300 and 1600 V/cm). Their results showed that sinusoidal pulses reduced both the extent of muscle contractions and skin damage. The effects were significantly lower when a high frequency wave was applied and when the square bipolar pulse was used. However, there was a clear loss of efficacy with the increase in frequency, confirming that the external electric field should be increased to 1600 V/cm in to achieve an equivalent EP effect, thus allowing for a tumour volume growth of less than 200 mm^2 within a 25 d follow-up period.

Reduction of muscle contraction and pain: Numerical analysis

Golberg and Rubinsky[28] performed a numerical analysis to evaluate the influence of the electrode geometry in the reduction of pain and muscle contractions. The numerical analysis considered various electrode configurations. For each experimental setup, a single pulse of 400 V and 100 μs was delivered. The results showed that conventional EP protocols and electrode design could generate muscle contraction, inducing electric fields in surprisingly large volumes of non-target tissue surrounding the EP-treated tissue. They also found that electrode placement in a structure referred to as a "current cage" substantially reduced the volume of non-target tissue exposed to electric fields above the threshold of muscle contraction. Furthermore, in an experimental study using a tissue phantom, they compared a commercial two parallel needle EP system with the current cage design. They found that a certain arrangement of needle electrodes limited the amount of tissue exposed to electric fields that above the muscle contraction threshold, while having a minimal impact on the extent of EP. The design consisted of a central, energised electrode surrounded by an array of grounded

electrodes. Similar geometries have been used successfully for cardiac defibrillation and ECT. Interestingly, by having 16 or more grounded electrodes and by reducing the insertion depth of the central energised electrode relative to the grounded electrodes, the predicted amount of tissue experiencing muscle contractions fell dramatically. In fact, the analysis revealed that the ratio of the volume affected by the muscle contraction (V_{mc}) and that affected by the EP phenomenon (V_{ep}) using a commercial parallel eight-electrode array, was 135 and was 410, with an electric field of than 600 V/cm and 1120 V/cm. The corresponding ratios were 73 and 26 when the 26-electrode current cage was used. Moreover, the total V_{mc} was 15.09 mm^2 when the commercial parallel eight-electrode array was used, compared with 2.90 mm^2 when using the 26-electrode current cage.

Reduction of muscle contraction and pain: IRE protocols

In 2011, Arena *et al*[32] used a combination of analytical, numerical and experimental techniques to investigate H-FIRE. In their *in vivo* protocols, they compared a standard IRE pulse protocol to H-FIRE. In both protocols, 180 bursts were delivered, with each burst lasting 200 μs and being delivered at a frequency of 1 Hz. In the IRE protocol, each burst consisted of a single pulse of 200 μs width. In the H-FIRE protocol, each burst consisted of (1) 50 bipolar pulses at 250 kHz and a single polarity duration of 2 μs; and (2) 100 bipolar pulses at 500 kHz with single polarity duration of 1 μs. No visual or tactile evidence of muscle contraction was seen during H-FIRE, but all IRE protocols resulted in detectable muscle contractions. The mean peak accelerations (0.8 *g*, 0.4 *g* and 0.1 *g*) during IRE treatments at the cervicothoracic junction for each applied voltage (200 V, 100 V and 50 V) were significantly different from each other. On the other hand, H-FIRE resulted in no detectable acceleration at the cervicothoracic junction. The *in vivo* experiments also showed that H-FIRE produced an ablative effect on brain tissue comparable to that obtained in non-thermal IRE treatments. Specifically, there was complete uniformity of tissue death within the targeted areas. A sharp transition zone was present between lesions and normal brain tissue.

In 2014, Sano *et al*[42] studied the effects of bipolar pulses on both muscle contractions and cell viability using an IRE protocol. Each monopolar waveform typical of the standard protocol was replaced with a burst of alternating polarity pulses; the total energised burst time was the same as that used in the standard protocol (100 μs). The bipolar protocol consisted of 80 bursts at a frequency of 1 Hz; in each burst, the positive/negative wavelength varied from 250 ns to 50 μs, with an intra-pulse delay fixed at 2 μs. The authors showed that, at 1500 V/cm, only treatments with bursts containing 50 + 50 μs pulses (Table 1) resulted in an interesting compromise between low viability (below 10%) and muscle contraction reduction that less undesirable than those associated with longer monopolar pulses. Sano *et al*[43]. analysed muscle contraction in a murine model when using different pulse protocols [43]. Treatment efficacy was also tested in an *in vitro* tumour model using PPT8182 murine primary pancreatic tumour cells. To facilitate comparison between groups, the authors applied the following simplified electrical dose formula:

$$Dose = V^2 \times T_p \times n \times N$$

Where V is the applied voltage, T_p is the pulse width, n is the number of pulses *per* burst, and N is the number of bursts *per* treatment, which was typically 80. Thanks to the use of a custom pulse generation system, bursts of bipolar pulses with constitutive pulse widths of 250 ns, 500 ns, 1 μs, 2 μs, 5 μs, 10 μs and 50 μs were delivered. They also used custom-made electrodes with 1.27 mm diameter dispensing needles and a 2.0 mm edge-to-edge separation distance. Given the formula above, the lethal electric field thresholds were found to be 2022, 1687, 1070, 755, 640, 629 and 531 V/cm for bursts containing 0.25, 0.5, 1, 2, 5, 10 and 50 μs pulses, respectively. Qualitatively, the results showed that muscle contractions occurred to a lesser extent in treatments with bipolar bursts of pulses between 1μs and 5 μs, compared with those in treatments with standard IRE protocol (100 μs monopolar pulses at 200 V). At 400 V, the 100-μs pulses induced such strong muscle contractions that complete anaesthesia was necessary to carry out the procedure. In contrast, 1000 V treatments with bursts of 5 μs pulses were well tolerated with light sedation and local anaesthesia.

Similarly, in 2016, Sweeney *et al*[33] carried out a quantitative comparison between different pulsing schemes. They compared trains of 100 μs monopolar pulses conventionally used in IRE and ECT, with pulse trains containing bursts or evenly spaced 1 μs bipolar pulses. They assessed both the reduction of muscle contractions and the cell permeability obtained with the different pulsed electric field protocols. Cell permeability was evaluated by real-time microscopic imaging of propidium iodide transport at the single cell level during and after each treatment. The protocols under investigation were: (1) A train of 200 monopolar pulses of 300 V amplitude, each

Table 1 Summary of the studies analysed in this review

Ref.		Electroporation protocol						Muscle contraction/pain reduction
			Type of pulse	Number of pulses	Pulse duration	Pulse frequency	Electric field	
Daskalov *et al* [36], 1999	*In vivo*	ECT	Monophasic	1 burst of 8 pulses	100 μs	1 Hz	0.33-1.25 kV/cm	Achievable with biphasic pulses
			Biphasic	1 burst of 8 pulses	50-0-50 μs	1 Hz		
			Biphasic	1 burst of 8 pulses	50-0-50 μs	Approximately 909 Hz		
Miklavcic *et al*[37], 2005	*In vivo*	ECT	Monopolar	1 burst of 8 pulses	100 μs	1 to 5000 Hz (ten or five steps)	88 or 313 V/cm	Achievable with high frequency pulses
Zupanic *et al* [38], 2007	*In vivo*	ECT	Monopolar	1 burst of 8 pulses	100 μs	1 Hz	600 V/cm	Achievable with high frequency pulses
			Monopolar	1 burst of 8 pulses	100 μs	5000 Hz		
Spugnini *et al* [39], 2014	*In vitro* *In vivo*	ECT	Biphasic	1 burst of 8 pulses	50-10-50 μs	9 kHz	1.3 kV/cm	Achievable with biphasic pulses
Scuderi *et al* [40], 2019	*In vitro*	ECT	Monopolar	1 burst of 8 pulses	100 μs	1 Hz	1.2 kV/cm	Achievable with bipolar HF-EP
			Bipolar	8 bursts of 50 pulses	1-1-1 μs	250 kHz	3 kV/cm	
García-Sánchez *et al* [41], 2020	*In vivo*	ECT	Unipolar	Bursts of 8 pulses	100 μs	1 Hz	1.3 kV/cm	Achievable with sinusoidal pulses
			Bipolar	Number of bursts and pulses depend on experiments and frequency	100 μs-5 ms	10-100 kHz	> 1.3 kV/cm	
			Sinusoidal					
Golberg and Rubinsky [28], 2012	*Numerical*	-	Monopolar	1 pulse	100 μs	-	> 800 V/cm	Achievable with an appropriate electrode design and arrangement
Arena *et al* [32], 2011	*In vivo*	IRE	Monopolar	90-180 pulses	200 μs	1 Hz	0.5-2 kV/cm	Achievable with H-FIRE
			Bipolar	180 bursts of 50 pulses	2-0-2 μs	250 kHz	1-4 kV/cm	
			Bipolar	180 bursts of 100 pulses	1-0-1 μs	500 kHz	4 kV/cm	
Sano *et al* [42], 2014	*In vitro*	IRE	Bipolar	80 bursts of 1 pulse	50-2-50 μs	1 Hz	1.5 kV/cm	Achievable with biphasic pulses
Sano *et al* [43], 2015	*In vitro*	IRE	Monopolar	1 pulse	100 μs	1 Hz	1.5 kV/cm	Achievable with bipolar pulses
	In vivo		Bipolar	8-120 bursts of 1-200 pulses	Pulse width: 250 ns-50 μs	20 to 20000 kHz (seven steps)	Approximately 0.5-2 kV/cm	
Sweeney *et al* [33], 2016	*In vitro*	IRE	Monopolar	1 burst of 200 pulses	100 μs	2 kHz	750-1250 V/cm	Achievable with high-frequency bipolar pulses
			Bipolar	200 bursts of 50 pulses	1 -1-1 μs	250 kHz	1250 V/cm	
			Bipolar	200 bursts of 50 pulses	1-4-1 μs	100 kHz	1250 V/cm	
Yao *et al*[44], 2017	*In vivo*	IRE	Bipolar	90 bursts of 50 pulses	2-2-2 μs	250 kHz	1-2 kV/cm	Achievable with H-FIRE and insulated needle electrodes
			Bipolar	90 bursts of 20 pulses	5-2-5 μs	Approximately 143 kHz		
			Bipolar	90 bursts of 10 pulses	10-2-10 μs	Approximately 83 kHz		
			Bipolar	90 bursts of 4 pulses	25-2-25 μs	Approximately 37 kHz	1–1.75 kV/cm	
			Bipolar	90 bursts of 2 pulses	50-2-50 μs	Approximately 20 kHz	1–1.5 kV/cm	
			Monopolar	90 bursts of 1 pulse	100 μs	1 Hz	0.8–1.5 kV/cm	
Sano *et al* [29], 2018	*Ex vivo*	IRE	Monopolar	5 pulses	100 μs	1 Hz	1 kV/cm	Achievable with (symmetric) H-FIRE pulses
			Bipolar	5 bursts of 1-200 pulses	2-2-2 μs	Not available	0.17-1.7 kV/cm	

ECT: Electrochemotherapy; H-FIRE: High-frequency irreversible electroporation; HF-EP: High frequency electroporation; IRE: Irreversible electroporation.

lasting 100 μs and repeated at a rate of 2 kHz for 500 μs; (2) A train with the same characteristics but with a pulse amplitude of 500 V; and (3) 200 bursts of 25 bipolar pulses with a 1 + 1 μs duration and 500 V, separated by a 4 μs intra- and inter-pulse delay. Each treatment consisted of 200 periods (bursts) lasting 500 μs each, for a total treatment time of exactly 100 ms for each pulsing scheme. Even though bipolar pulses at a high frequency were able to mitigate undesirable muscle contraction during IRE, the bipolar pulses induced less evident membrane permeabilisation than equivalent monopolar pulses. In fact, intracellular detection of propidium iodide was observed at electric field intensities of approximately 500 V/cm, which was lower than that observed in bipolar pulse treatments (900-1250 V/cm). That was attributed to the inability of the short-duration bipolar pulses to complete the membrane charging despite the higher applied voltages. However, bipolar pulse protocols can be designed to obtain more efficient, symmetric and homogeneous uptake of small molecules into cells than conventional monopolar pulses.

In 2017, Yao *et al*[44] explored the effect of IRE ablation on muscle contractions. The authors studied how to reduce muscle contractions by acting both on the frequency of monopolar pulses and on the nature of the electrodes used. The study was conducted with rabbit liver tissue. The H-FIRE protocol consisted of a series of 90 bursts. Each burst had a repetition frequency of 1 Hz and comprised 50, 20, 10, 4 or 2 monopolar pulses with individual pulse widths of 2, 5, 10, 20 or 50 μs. The total energised time was 100 μs. The experiments were conducted with both traditional and insulated needle electrodes with the aim of investigating how the electrode design influenced the muscle contractions. Each pair of electrodes were separated by a fixed distance of 10 mm. A finite element model was also used to establish the lethal thresholds of H-FIRE protocols; consequently, the pulse voltage amplitude range was set from 800 V to 2000 V. An accelerometer was used to measure muscle contractions. The authors observed that the H-FIRE protocol reduced muscle contractions. The muscle contraction strength increased with the increase in voltage amplitude and pulse width. A quite linear increase in acceleration occurred when the voltage was increased, regardless of the pulse duration. For example, a 10 μs pulse produced an acceleration of about 1.5 *g* at 1000 V, and about 4 *g* at 2000 V Conversely, at a fixed voltage, a consistent increase in the acceleration value was observed when the pulse length was also increased (*e.g.*, less than 1 g of acceleration for 2 μs pulses at 1500 V *vs* more than 7 *g* of acceleration for 100 μs pulses at 1500 V). Moreover, fewer muscle contractions were detected when using insulated needle electrodes and the ablation area was smaller than that obtained with traditional needle electrodes (*e.g.*, about 5 *g* of acceleration with 50 μs pulses at 1500 V using insulated needles *vs* about 6.5 *g* of acceleration with 50 μs pulses at 1500 V using non-insulated needles).

Sano *et al*[29] compared the effect on muscle contraction associated with IRE to those associated with different H-FIRE protocols. The experiments were conducted *ex vivo* and muscle contractions were measured with an accelerometer. In order to make the comparison consistent, the total energised time in H-FIRE protocols was ensured to be equal to one of the standard IRE protocols. The traditional IRE protocols consisted of five monopolar pulses lasting 25, 50, 75 and 100 μs, with a repetition frequency of 0.5 or 1 Hz and with an amplitude of 3000 V. To examine alternative strategies, high-energy bipolar bursts with energised times between 100 μs and 200 μs and voltages between 3000 V and 4500 V were delivered. The investigated H-FIRE protocols were split into three subgroups: (1) Symmetric 2 + 2 μs high frequency pulses with an intra-pulse delay of 2 μs, voltages of from 500 to 5000 V and total energised times of 100 or 200 μs; (2) Symmetric 2 + 2 μs high frequency pulses with an intra-pulse delay of 5 μs or 10 μs, a voltage of 5000 V and a total energised time of 100 μs; and (3) Asymmetric high frequency pulses with a 2 μs positive wave, an intra-pulse of 2 μs and negative waves of 0.25, 0.5 or 1 μs (voltage of 3000 V). An energised time of 100 μs with 2–2–2 H-FIRE pulses produced muscle contractions that increased with the voltage (accelerations of 0.005 *g* and 0.210 *g* for voltages of 500 and 5000 V, respectively). When the voltage was set at 3000 V, the acceleration peak obtained in the symmetrical H-FIRE protocol was 9-12 times smaller than that seen with traditional IRE pulses (0.72 *g* with a 75 μs pulse length). Moreover, symmetrical high frequency pulses enabled the delivery of substantially higher voltages and energised times while producing smaller accelerations than traditional IRE pulses. In fact, the acceleration values remained relatively constant when the total energised time was increased from 100 μs to 200 μs, and even when the applied voltage was increased to 4500 V. Conversely, both

symmetric pulses with a variable intra-pulse delay and asymmetric pulses produced significantly greater muscle contractions. However, asymmetrical H-FIRE produced significantly greater ($\alpha < 0.001$) muscle contractions at 3000 V compared with the symmetrical waveforms. The maximum peak acceleration (0.80 *g*) comparable to that achieved with the traditional IRE pulses, was achieved with the 2–2–0.25 waveform. The authors concluded that muscle contractions can be reduced with H-FIRE pulses when the voltage and energised time are held constant (3000 V, 100 μs). Additionally, high voltage and high-energy H-FIRE treatments produced less intense muscle contractions. However, since the experiments were conducted *ex vivo*, it is reasonable to consider that muscle contractions *in vivo* may be greater than those observed in this study. Ablation efficacy should also be assessed.

Summary table

Table 1 summarises the outcomes of the literature analysis. The type of study (numerical analysis, *in vitro*, *in vivo* or *ex vivo*), the type of electroporation protocol (ECT or IRE), the pulse characteristics and the main results are reported. With reference to the pulse characteristics, it was considered of particular interest to report: (1) The type of pulse; (2) The number of pulses (*i.e.* the number of bursts and the number of pulses *per* burst); (3) The pulse duration (when bipolar/biphasic, as positive pulse width–intra-pulse delay–negative pulse width); (4) The pulse frequency (*i.e.* the inverse of a single monopolar/monophasic or bipolar/biphasic pulse period); and (5) The electric field applied (Figure 3).

DISCUSSION

Muscle contractions and pain are the main undesirable effects associated with EP treatments, both ECT and IRE. Many authors have investigated different protocol designs and electrode geometries in order to reduce patient pain, the number of muscle contractions and their intensity. As this review shows, particular importance was given to the length, frequency and type of the delivered pulses. Less attention was paid to the influence of the electrode design even though it does affect the portion of the muscle through which the current flows. As reported by Miklavcic *et al*[37] the reduction of voltage amplitude, does not result in an appreciable reduction of patient discomfort. Moreover, even if it is widely accepted that a decrease in the pulse amplitude can be balanced by an increase in the pulse duration, there is still disagreement as to how to determine the correct increase in the number of pulses to be applied in order not to alter treatment efficacy.

Pain reduction and pulse frequency

The reduction of pain due to muscle contractions can be obtained by increasing the pulse repetition frequency above that of tetanic contraction (100 Hz)[38]. When the frequency was higher than 2 kHz, patients experienced a single muscle contraction rather than multiple muscle contractions after every single pulse[38,39]. Moreover, treatment efficacy was not altered by an increase in frequency of up to 5 kHz[38,39]. That was confirmed by Yang *et al*[47], who observed that steep pulsed electric fields with a given frequency and appropriate electric field intensity achieved a cytotoxicity of close to 100%. However, even though the total number of muscle contractions *per* treatment was reduced, the intensity of the contractions remained similar to that observed in standard protocols.

When altering the pulse repetition frequency, attention should be paid to the choice of pulse numbers and amplitude[48,49]. In fact, the relationship between the pulse parameters for ECT and treatment efficacy, assessed by the cell cytotoxicity rate, can display a highly linear behaviour up to a certain number of pulses and/or field intensity. Thereafter, an exponential model is more appropriate. That is consistent with a recent study by García-Sánchez *et al*[41], which found that convenient, reversible EP and efficient ECT of subcutaneous tumours and a remarkable reduction of muscle contraction could be achieved by applying sinusoidal fields. However, the frequency of sine waves has been shown to significantly affect ECT effectiveness. At 100 kHz, a clear loss of efficacy was observed[41]. In order to achieve a tumour regression similar to that obtained at 10 kHz, the electric field intensity should be theoretically increased 1.56 times. Those results highlight the charge-dependent nature of the EP phenomenon, where the cell membrane must be charged at the minimum induced transmembrane voltage in order to achieve effective electro-permeabilisa-tion [49,50].

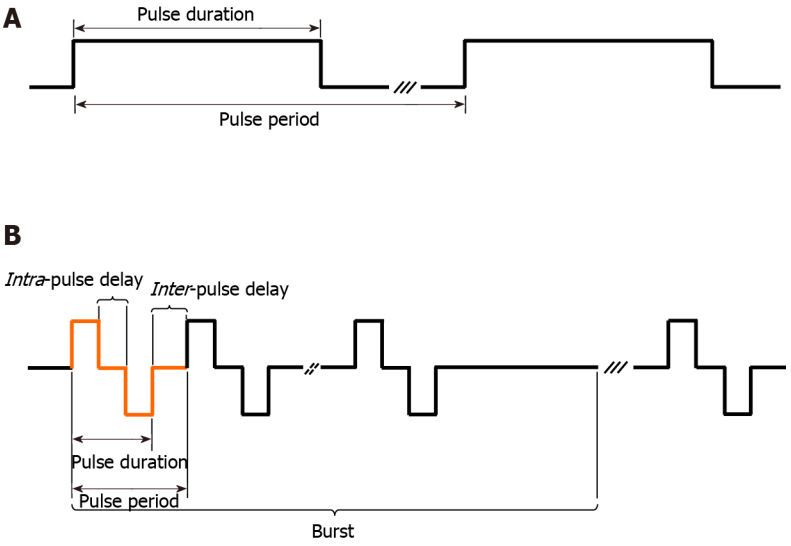


Figure 3 Pulse structure. A: Monopolar/monophasic pulse; B: Bipolar/biphasic pulse.

Pain reduction and bipolar/biphasic pulses

Several authors have investigated reduction in morbidity achieved with bipolar/biphasic pulses. They all reported that altering the pulse polarity not only reduced the occurrence but also the intensity of muscle contractions. In fact, when a μs-pulse was applied, there was a latency period between the end of the pulse and the rising phase of the action potential. A rapid reversal of polarity within this latency period can accelerate passive repolarisation and inhibit the action potential generation. Therefore, with proper tuning of the bipolar/biphasic pulse parameters, it is possible to achieve a drop in muscle force that can be attributed to the termination of action potentials in part of the motor unit population[33,34,40,43,44,48,51], without losing in EP efficacy. With that in mind, interesting results were reported by Spugnini *et al*[39]. They reported that trains of eight biphasic electric pulses lasting 50 + 50 μs each, at a frequency of 1 Hz, and 10 μs interpulse intervals at 1300 V/cm, achieved a significantly higher response in mice (70%-90% tumour necrosis) compared with that achieved with trains of eight biphasic electric pulses lasting 50 + 50 ms each, at a frequency of 1 Hz and 1-ms interpulse intervals (40%-55% tumour necrosis)[40].

Sano *et al*[43] reported that bursts of bipolar pulses resulted in both instantaneous and delayed cell death and that an inverse relationship existed between pulse width and toxicity, despite the delivery of equal quantities of energy. However, 1500 V/cm bursts containing 50 + 50 μs pulses resulted in a viability below 10% and low muscle contractions, which was less undesirable than those induced by longer monopolar/monophasic pulses. This result is comparable with the standard IRE protocol, as reported by Arena *et al*[51], who showed that after eighty 100 μs monopolar pulses at 1500 V/cm, cell viability was approximately 8%. Bipolar/biphasic pulses in the same electric field seem to have appreciable efficacy when biphasic pulses of 50 + 50 μs are used; however, in order to obtain a comparable cytotoxicity rate with bipolar/biphasic and monopolar/monophasic protocols, the bipolar/biphasic pulses generally need a stronger electric field[41].

Finally, the protocols described by Scuderi *et al*[40] and Sweeney *et al*[33] achieved the results obtained with high frequency pulses together with those achieved with bipolar pulses in ECT and IRE. They found that bipolar pulses at a high frequency were able to mitigate both undesirable muscle contraction and patient pain in EP therapies. Additional reduction was achieved when the bipolar pulse had a symmetrical structure[30]. However, short bipolar pulses may result in less pronounced membrane permeabilisation, suggesting that pulse duration is a critical parameter that must be carefully chosen[34,50].

Pain reduction and electrode design

Reductions of muscle contraction and morbidity can be achieved with an appropriate electrode design. Fewer and less intense muscle contractions were reported by Yao *et al*[44] when using insulated needle electrodes. A more sophisticated electrode design was proposed by Golberg and Rubinsky[28] where a central energised electrode was surrounded by at least 16 grounded electrodes, obtaining significant pain reduction.

However, the impact of the new electrode designs on treatment efficacy remains to be more deeply evaluated.

CONCLUSION

This review found that both pulse frequency and shape can be modified to reduce pain and muscle contractions in patients who undergo EP treatments. Furthermore, a combination of high frequency pulses with bipolar/biphasic ones were shown to enhance this capability. However, simply ensuring that equivalent energy is administered by standard and new protocols (high frequency, bipolar/biphasic) is not sufficient to guarantee clinical efficacy. Preclinical *in vitro* and *in vivo* studies together with clinical data are necessary to evaluate the clinical relevance of alternative pulse protocols. In addition, sinusoidal pulses with the appropriate frequency, as well as electrode design (*e.g.*, insulated needles), may successfully mitigate these drawbacks. Further study is required to evaluate how these aspects influence the efficacy of the therapy. A main limitation of this systematic review is the absence of a risk of bias analysis both in individual studies and across studies. Moreover, the authors did not investigate principal summary measures (*e.g.*, risk ratio, difference in means, and others), as most studies did not report numerical results. Those deficiencies should be addressed in subsequent investigations. To summarise, pain reduction in EP-based treatments can be achieved by appropriately defining the protocol parameters and the electrode design. The desired results can be achieved with high frequency and/or bipolar/biphasic pulses. However, the efficacy of these alternative protocols remains a crucial point to be assessed further.

ARTICLE HIGHLIGHTS

Research background

In electrochemotherapy (ECT), pain and muscular contractions were reported as the most frequent drawbacks.

Research motivation

To review aimed to assess the literature describing technical advances intended to reduce muscle contraction and pain associated with electroporation (EP) effects.

Research objectives

The objective was to present an overview of different protocols proposed in the literature that aim to reduce muscle contraction in both ECT and irreversible EP treatments.

Research methods

Thirty-three published studies reporting different protocol designs and electrode geometries were selected for analysis.

Research results

Both high frequency and bipolar/biphasic pulses can be used to reduce pain and muscle contractions in patients who undergo EP treatments. Moreover, adequate electrode design can lower EP-related morbidity.

Research conclusions

Pain reduction in EP-based treatments can be achieved by appropriately defining the protocol parameters and the electrode design.

Research perspectives

The desired results can be achieved with high frequency and/or bipolar/biphasic pulses.

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SYSTEMATIC REVIEWS

Intestinal metastasis from breast cancer: Presentation, treatment and survival from a systematic literature review

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Abstract

BACKGROUND

Intestinal metastases from breast cancer (BC) arerare; available data depend mainly on case reports and case series.

AIM

To conduct a review of the literature regarding presentation, diagnosis, treatment and survival of patients with intestinal metastasis from BC.

METHODS

We identified all articles that described patients with intestinal metastasis (from duodenum to anum) from BC using MEDLINE (1975 to 2020) and EMBASE (1975 to 2020) electronic databases.

RESULTS

We found 96 cases of intestinal metastasis of BC. Metastasization involved large bowel (cecum, colon, sigmoid, rectum) (51%), small bowel (duodenum, jejunum, ileum) (49%), and anum (< 1%). Median age of patients was 61-years. The most frequent histology was infiltrating lobular carcinoma followed by infiltrating ductal carcinoma. In more than half of patients, the diagnosis was made after the diagnosis of BC (median: 7.2 years) and in many cases of emergency, for bowel

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obstruction, bleeding or perforation. Diagnosis was achieved through endoscopy, radiological examination or both. In most of the cases, patients underwent surgery with or without systemic therapies. Survival of patients included in this review was available in less than 50% of patients and showed an overall median of 12 mo since diagnosis of the intestinal metastasis.

CONCLUSION
Although, intestinal metastases of BC are considered a rare condition, clinicians should consider the possibility of intestinal involvement in case of abdominal symptoms even in acute setting and many years after the diagnosis of BC, especially in patients with a histology of lobular carcinoma.

Key Words: Breast cancer; Intestinal metastasis; Diagnosis; Treatment; Small bowel; Large bowel

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Core Tip: We conducted a review of the literature regarding presentation, diagnosis, treatment and survival of patients with intestinal metastasis from breast cancer (BC). Although intestinal metastases of BC are considered a rare condition, several cases are reported from the available literature. Clinicians should consider the possibility of intestinal involvement in case of abdominal symptoms even in acute setting and many years after the diagnosis of BC.

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INTRODUCTION

Breast cancer (BC) is the most common malignancy among women and a leading cause of cancer-related deaths[1,2]. In case of early diagnosis and application of new therapies, approximately 30% of patients are still at risk of developing distant metastasis, while 5% of the cases are metastatic at diagnosis[3]. The most common metastatic sites of BC are bones, lungs, liver and brain[4]. Gastrointestinal (GI) tract metastases are uncommon, detected in less than 5% of all BC patients[5]. Distinguishing primary and secondary GI cancer tumors can be clinically challenging. In particular, intestinal metastases from BC are rare, and related symptoms are not specific and often attributed to oncologic treatment, the main problem is to recognize them promptly and discriminate peritoneal carcinomatosis from other GI diseases to avoid any diagnostic delay and establish an effective treatment as soon as possible to improve survival of patients[6].

Despite well-known potential of intestinal metastatization of the BC, available data rely mainly on case reports and case series. Therefore, we report a literature review on presentation, diagnosis, treatment and survival of patients with intestinal metastasis from BC.

MATERIALS AND METHODS

We identified all articles that described patients with intestinal metastasis (from duodenum to anum) from breast cancer using MEDLINE (1975 to 2020) and EMBASE (1975 to 2020) electronic databases.

The search strategy was developed with a language restriction (only English texts) and literature search performed by applying the words: "Breast cancer", "gastrointestinal metastasis", "gastrointestinal tract", "intestine, bowel", "duodenum", "jejunum, cecum", "ileum", "small bowel", "large bowel", "colon", "sigma", "sigmoid

tract", "rectum", "anus" using the following string [(breast) AND (cancer or tumor or neoplasm) AND [duodenal neoplasms (secondary) OR jejunal neoplasms (secondary) OR cecal neoplasms (secondary) OR ileal neoplasm (secondary), OR small bowel neoplasm (secondary), OR colorectal neoplasms (secondary) OR sigma OR sigmoid neoplasm (secondary) OR anal neoplasms (secondary) NOT review], and filtering them for English studies and for humans studies (Figure 1).

The research of the literature was performed independently by two investigators (Nigro O and Bolzacchini E).

Studies were included if they met the following criteria: (1) Patients ≥ 18 years; (2) Patients with intestinal metastasis from breast cancer; and (3) Diagnosis of intestinal metastasis was objectively confirmed (histology).

Two investigators (Nigro O and Bolzacchini E) independently extracted data on study (year of publication, study centre), patients' characteristics (number of subjects studied, age, gender), tumour's characteristics (histology, time and site of metastatization), clinical presentation (main symptoms reported) as well as treatment and survival from the diagnosis of intestinal metastasis. We tried to contact the authors of the articles with missing survival data.

RESULTS

We identified 96 cases (86 articles) of intestinal metastases from BC[5-91]. Metastases are described in all parts of the intestinal tract, from the duodenum to the anum. Site of metastasis, presentation symptoms, treatment and clinical data are reported in Table 1.

Metastatization arose in large bowel (cecum, colon, sigmoid, rectum) (50/96; 52%), small bowel (duodenum, jejunum, ileum) (47/96; 49%), and anum (4/96, < 1%). Four patients presented multiple sites of intestinal metastases (small and large bowel); in three patients, gastric metastasis was also found, while peritoneum was also involved in six patients. Median age of patients was 61-years (between 31 and 88-years-old); only two patients were males. Histology comprehended lobular carcinoma (56/96; 58%), ductal carcinoma (17/96, 18%), phyllodes tumor (3), tubular carcinoma (1), or mixed histology (6).; 13/96 histologies were unknown. Intestinal involvement was diagnosed after the diagnosis of BC in 59/96 patients (median time; 7.2 years; range: 3 mo-25 years); the diagnosis was concomitant in 20/96 patients, in one case, the diagnosis of BC was made months after the metastatic involvement of the intestine and in another case, BC remained occult. In many cases, the diagnosis was made in emergency, for bowel obstruction (39 patients, 40.6%), bleeding (10 patients, 10.4%) and perforation (2 patients, 2%). Other patients complained of symptoms such as pain, changes in bowel habits, and in few patients, the diagnosis was incidental.

Diagnosis was achieved through endoscopy (esophagogastroduodenoscopy, colonoscopy or video capsule enteroscopy) in 54/96 cases (56.2%), radiological examination [computed tomography (CT), magnetic resonance imaging, barium enema or positron emission tomography (PET)] in 82/96 cases (85.4%) or both endoscopy and radiological imaging in 44/96 cases (45%).

In most of the cases, patients underwent surgery (69/96; 72%), with or without systemic therapies. The other patients started or continued medical therapy (18/96, 18.7%) such as hormone therapy and chemotherapy.

Specifically, 40 patients received hormone therapy (one patient aromatase inhibitor plus palbociclib), 38 patients received chemotherapy and 9 patients received both. Chemotherapy prescribed included monotherapy agents such as taxane-based chemotherapy (paclitaxel, docetaxel), anthracycline-based and oral fluoropyrimidine. Moreover, chemotherapy regimen was not specified in many reports.

Median overall survival of patients included in this review was available for 46/96 pts (< 50%); median survival estimated from the available data was around 12 mo.

DISCUSSION

Intestinal metastases from BC are rare. Jain *et al*[92] in a large study examined 1238 patients with operable BC and identified metastatic sites. They found that infiltrating ductal carcinoma recurred more often in the lung, pleura and bone, while infiltrating lobular carcinoma metastasized more often to bone marrow and peritoneum. Bone involvement as the initial presentation of distant metastatic disease occurred in over 50% of the women with infiltrating lobular carcinoma, significantly more often than in

Table 1 Demographic and clinical data of patients with intestinal metastasis from breast cancer

	n (%)	Median (range)
Year of publication		
< 2000	5 (5.8)	
2001-2005	12 (13.9)	
2006–2010	18 (20.9)	
2011-2015	36 (41.8)	
> 2016	15 (17.4)	
Age		61 (31-88)
Gender		
Female	94 (97.9)	
Male	2 (2.1)	
Breast cancer histology		
Lobular carcinoma	56(58.3)	
Ductal carcinoma	17 (17.7)	
Phyllodes tumor	3 (0.3)	
Tubular carcinoma	1 (0.1)	
Mixed histology	6 (0.6)	
Unknown	13 (13.5)	
Time of the diagnosis		
After the diagnosis of breast cancer	59 (61.4)	
Concomitant	20 (20.8)	
Before	1 (0.2)	
Unknown	16 (16.6)[1]	
Time after the diagnosis of breast cancer (yr)		7.2 (3 mo-25 yr)
Clinical presentation or main symptom at diagnosis		
Bowel obstruction	39 (40.6)	
Rectal bleeding	10 (10.4)	
Perforation	2 (2)	
Abdominal pain	20 (20.8)	
Change in bowel habit	9 (9.3%)	
Incidental	12 (12.5)	
Unknown	4 (4.1)	
Site of metastatization		
Colon	45 (46.8)	
Small bowel	43 (44.8)	
Colon and small bowel	4 (4)	
Anus	4 (4)	
Diagnostic tool for the detection of the metastasis		
Endoscopy (EGDS or colonscopy or VCE)	54 (56.2)	
Radiography (CT scan, MRI, barium enema, PET)	82 (85.4)	
Unknown	12 (12.5)	
Treatment		

Surgery and medical therapy	69/96 (71.8)	
Hormone therapy and/or chemotherapy	18/96 (18.7)	
Survival after the diagnosis of metastasis (yr)[2]		12 (1 mo-7 yr)

[1]In one case the primary tumor remained occult.
[2]Available for 46 patients. EGDS: Esophago-gastro-duodenoscopy; VCE: Video capsule endoscopy; CT: Computed tomography; MRI: Magnetic resonance imaging; PET: Positron emission tomography.

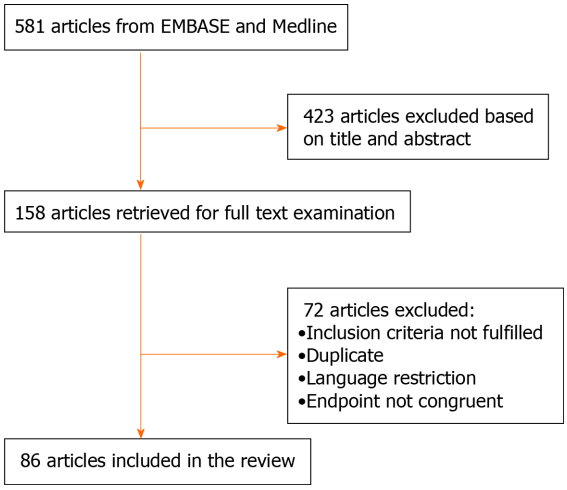


Figure 1 Study identification and selection process.

those with ductal infiltrating carcinoma (34%; $P < 0.01$). Survival was similar for the 2 groups.

Interestingly, metastatic disease to the extrahepatic GI tract from breast carcinoma usually originates from the lobular carcinoma subtype, which accounts for only 8% to 14% of all breast adenocarcinomas, rather than the more common invasive ductal carcinoma[93]. This could be related to a particular tropism of lobular cells. In a large autopsy series[94] of 337 patients who died of BC, GI metastasis presented an incidence of 16.4 % and only 20% of these patients complained of having symptoms. However, in this autopsy series, gastric and intestinal metastases were grouped, preventing an accurate estimation of the latter.

It has been well documented that recurrence in lobular BC can occur several years after the initial diagnosis of BC, even in early stage tumors. Recurrences of lobular BC have been reported up to 30 years from the initial time of diagnosis[95]. Accurate incidence of GI metastasis is hard to establish.

Symptoms depending on metastatic sites are generally not specific and may include abdominal pain, dyspepsia and nausea, acute symptoms such as bowel occlusion and GI bleeding may occur in most cases. Time interval between primary BC cases is wide, mostly years after the first diagnosis but rarely even before.

Endoscopy, radiological exams and histological evaluation are necessary to differentiate primary GI carcinoma from metastatic GI lesion of BC[32].

Endoscopic findings vary significantly and may range from ulcers, mucosal thickening or friability, linitisplastica-like inflammation, stenosis and polyps, to obstructing mass[24]. Barium studies reveal stricture or linitisplastica[41].

CT is indicated for characterizing the extension of the mass and for the re-staging [96]. Recently, Laoutliev *et al*[9] suggested that 18F-fluorodeoxyglucose PET should be considered.

Markers are generally not useful nor specific for the diagnosis of intestinal metastasis, but in an interesting case report by Santini *et al*[8] an increase in CA19.9 was used to diagnose ileocaecal valve metastasis from BC.

Differentiation of breast metastasis from other GI primaries can be difficult and immunohistochemistry is crucial to establishing the accurate diagnosis. Commonly used markers include estrogen receptors and progesterone receptors, CK7, CK20. A CK7-/CK20+ profile favors a large bowel primary, while CK7+/CK20- favors a metastasis[74].

GCDFP-15 and mammaglobin positivity was found to be sensitive and specific markers were used to differentiate a malignant lesion as a metastatic breast carcinoma, with an excellent correlation between GCDFP-15 and mammaglobin positivity and the origin of a metastatic BC[97,98]. It is very important to clarify the diagnosis since the treatment strategy for GI metastasis of BC and for primary GI carcinoma is totally different[99].

However, data on treatment are fragmentary. Surgical treatment, often palliative, should be reserved in case of emergencies (perforation, obstruction and hemorrhage), patients with isolated lesions, and selected cases in which tumor debunking could improve clinical outcome. Medical treatment such as chemotherapy, hormone therapy and anti HER2 therapy may be indicated depending on the biologic features of the primary disease and on prior therapy[100] and it is recommended as first-line treatment in case of multiple metastatic involvement. These therapies can be extremely effective and may help to avoid unnecessary surgery[10]. Radiotherapy is an option in case of anal localization[26,57] and in case of brain metastases[33] or bone metastases.

Long term outcome remains uncertain due to data scarcity and for the rareness of the condition. Nevertheless, some cases of longer survival were also reported[6,30]. Considering the available data, intestinal involvement seems prognostically unfavorable; in fact, progression free survival and overall survival of patients affected with metastatic BC have improved over the years thanks to the new treatment options.

We are aware that this review includes a selection bias as it does not rely on consecutive series of patients, but on many selected single case reports, published in English over a wide lag time (30 years). Therefore, many cases may not have been reported, several clinical data are missing and the outcome is especially not available for many cases even though we tried to contact the authors.

We pointed out that our review includes cases published from 1975 till date and clinical presentation, diagnosis and therapy might have changed over the years with awareness and technological advancement, nuclear medicine and new therapies. In this regard, a tentative analysis to assess the influence of time on several diagnostic and prognostic variables has been performed (data not shown), but no significant difference was found.

Nevertheless, the strength of our paper is its originality and the systematic methodological approach to the literature regarding intestinal localization of metastatic BC. Several reviews have already been published regarding gastric metastasis of BC; but to the best of our knowledge this is the first regarding intestinal involvement. Our paper comprehends all the cases reported so far in English language and summarizes epidemiology, symptoms, diagnostic work-up, therapy and survival of this condition. On this note, an estimate of the problem is given to the best of its knowledge and it forms the basis for the creation of a multicentre prospective study or a registry, which is the best option to investigate this uncommon but relevant issue of BC.

CONCLUSION

Clinicians should consider the possibility of intestinal involvement in case of abdominal symptoms, especially in patients with a histology of lobular carcinoma. Adequate imaging and endoscopic procedure should be performed promptly in order to obtain histological diagnosis. Treatment strategies include surgery, chemotherapy, hormone therapy and radiotherapy. Long term prognosis remains uncertain.

A multicenter study or registry study is required.

ARTICLE HIGHLIGHTS

Research background

Intestinal metastasis from breast cancer (BC) is considered rare.

Research motivation

We conducted a review of the literature regarding intestinal metastasis from BC.

Research objectives

We conducted a review of the literature regarding presentation, diagnosis, treatment and survival of patients with intestinal metastasis from BC.

Research methods

We identified all articles that described patients with intestinal metastasis from BC using MEDLINE and EMBASE electronic databases until 2020.

Research results

We found 96 cases of intestinal metastasis of BC. Metastasization involved large bowel in 51% of the cases, small bowel in 49% of the cases, and anum in less than 1%. Median age of patients was 61-year-old. The most frequent histology was infiltrating lobular carcinoma followed by infiltrating ductal carcinoma. In more than half of patients the diagnosis was made after the diagnosis of BC and in many cases in emergency setting, for bowel obstruction, bleeding or perforation. Diagnosis was achieved through endoscopy, radiological examination or both. In most of the cases patients underwent surgery with or without systemic therapies.

Research conclusions

Although intestinal metastases of BC are considered a rare condition, several cases are reported from the available literature.

Research perspectives

Our paper comprehends summarizes epidemiology, symptoms, diagnostic work-up, therapy and survival of this condition. On this account it gives an estimate of the problem and could lead to and represent the basis for the creation of a multicentre prospective study or a registry.

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OPINION REVIEW

Health Council of the Netherlands and evaluation of the fifth generation, 5G, for wireless communication and cancer risks

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Abstract

Currently the fifth generation, 5G, for wireless communication is about to be rolled out worldwide. Many persons are concerned about potential health risks from radiofrequency radiation. In September 2017, a letter was sent to the European Union asking for a moratorium on the deployment until scientific evaluation has been made on potential health risks (http://www.5Gappeal.eu). This appeal has had little success. The Health Council of the Netherlands released on September 2, 2020 their evaluation on 5G and health. It was largely based on a World Health Organization draft and report by the Swedish Radiation Safety Authority, both criticized for not being impartial. The guidelines by the International Commission on Non-Ionizing Radiation Protection were recommended to be used, although they have been considered to be insufficient to protect against health hazards (http://www.emfscientist.org). The Health Council Committee recommended not to use the 26 GHz frequency band until health risks have been studied. For lower frequencies, the International Commission on Non-Ionizing Radiation Protection guidelines were recommended. The conclusion that there is no reason to stop the use of lower frequencies for 5G is not justified by current evidence on cancer risks as commented in this article. A moratorium is urgently needed on the implementation of 5G for wireless communication.

Key Words: 5G; Cancer risk; Health Council Netherlands

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Core Tip: In this comment, guidelines for radiofrequency radiation are discussed in

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relation to a recent evaluation by the Health Council of the Netherlands. The Committee recommends that for the deployment of 5G the frequency band 26 GHz should not be used. For lower frequencies, the International Commission on Non-Ionizing Radiation Protection guidelines are recommended. However, these guidelines are not based on an objective evaluation of health risks, which is discussed in this paper.

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INTRODUCTION

The fifth generation, 5G, for wireless communication is about to be rolled out worldwide in spite of health concerns. This has created debate among concerned people in many countries. In an appeal to the European Union (EU) in September 2017, currently endorsed by more than 400 scientists and medical doctors, a moratorium on the 5G deployment was required until proper scientific evaluation of negative consequences has been made (http://www.5Gappeal.eu). This has not had any impact on the progress of the deployment of 5G.

On September 2, 2020, the Health Council of the Netherlands released their evaluation of 5G and health (No. 2020/16/16e/16Ae). The Committee consists of 9 members, 2 scientific secretaries, 1 incidentally consulting expert, and 3 observers. (https://www.healthcouncil.nl/documents/advisory-reports/2020/09/02/5g-and-health).

Of large concern as to impartiality is that one member of the Committee, Huss A, is a member of the International Commission on Non-Ionizing Radiation Protection (ICNIRP) since May 2020. ICNIRP is a private organization based in Germany that does not acknowledge health effects other than caused by heating from radiofrequency (RF) radiation. Thereby nonthermal biological effects are disregarded.

Furthermore, one of the two secretaries, van Rongen E, has been a long-time commission member of ICNIRP since 2010, chair 2016 to 2020, and vice chair since May 2020 (https://www.researchgate.net/profile/Eric_Rongen). A third former ICNIRP member, Sienkiewicz Z, participated as a consulting expert.

It should be added that Huss A receives research funding from a telecom industry sponsored Swiss Foundation, and she is also member of this foundation's Scientific Committee (https://www.emf.ethz.ch/en/foundation/organisation). Further, van Rongen E is a long-time member of the industry organizations, the Institute of Electrical and Electronics Engineers and the International Committee on Electromagnetic Safety (IEEE/ICES).

These facts give concern of conflicts of interest in the Health Council Committee. Members of ICNIRP tend to adhere also in other settings to the ICNIRP no-risk paradigm regarding nonthermal RF radiation. This may also be the case in the Health Council report. In fact it should be noted that the Ethical Board at the Karolinska Institute in Stockholm, Sweden concluded already in 2008 that being a member of ICNIRP may be a conflict of interest that should be stated officially whenever a member from ICNIRP makes opinions on health risks from electromagnetic fields (EMFs) on behalf of another organization, as in this case (Karolinska Institute Diary No. 3753-2008-609). This verdict is related to Prof. Ahlbom A, ICNIRP Commission Member from 1996 until 2008, and is a general statement. Those involved in the current Health Council report with ICNIRP affiliation (present or former) omitted to state that conflict of interest.

Recommendations by the Health Council of the Netherlands

The Committee has made four recommendations to the Parliament, cited in the following: (1) Because the lower frequency bands for 5G (up to 3.5 GHz) have already been used for telecommunication applications and Wi-Fi for years without resulting in any proven adverse health effects, the committee sees no reason to stop or restrict the use of these frequency bands. It does, however, recommend that the exposure should

be monitored before, during and after the rollout of the 5G systems. This will make clear to what extent exposure to radiofrequency EMF changes as a result of the introduction of 5G, and any long-term health risks can then be estimated better. The World Health Organization (WHO) analysis can also be used in estimating the risks; (2) The committee recommends doing more research: epidemiological research into the relationship between exposure to the 5G frequencies used and the incidence of cancer, reduced male fertility, poor pregnancy outcomes and birth defects. An ongoing international study into the use of mobile telephones, in which the Netherlands is participating, can play a role in this: (a) experimental research into the health effects of exposure to EMFs in the 26 GHz frequency band; (b) scenario studies to get a picture of the exposure of individuals as a result of wireless communication systems (3G, 4G and 5G); (3) The committee recommends to not use the 26 GHz frequency band for 5G for as long as the potential health risks have not been investigated; and (4) Finally, the committee recommends using the latest guidelines from the ICNIRP as the basis for exposure policy in the Netherlands. Because it cannot be excluded that exposure under the latest ICNIRP standards also has the potential to affect health, the committee recommends taking a cautious approach and keep exposures as low as reasonably achievable.

Of these conclusions, number 3 is in agreement with an appeal to the EU asking for a moratorium on the roll-out of 5G until research has been done on potential health risks, although the appeal concerned all 5G frequencies (https://www.5gappeal.eu). The appeal has currently been signed by more than 400 international scientists and/or medical doctors.

The claim that "the lower frequency bands for 5G (up to 3.5 GHz) have already been used for telecommunications applications and Wi-Fi for years without resulting in any proven adverse health effects" is incorrect.

Evidence from research on cells, animals and humans shows that the frequencies used so far for telecommunications are harmful for humans and the environment. For overviews see[1-3].

There is no substantial research on health effects from the frequencies in the range between 3 and 4 GHz intended to be used for 5G as noted by the French authority ANSES in an expert report published in January 2020.

The Health Council Committee in Netherlands has mainly based its review on a 2014 draft report from the WHO and the reports from the Swedish Radiation Safety Authority (SSM). Huss A and van Rongen E have been coauthors of the Swedish SSM reports, and van Rongen E was furthermore a member of the WHO draft report's core group of members, see Table 1.

The Committee recommends using the latest ICNIRP 2020 guidelines, which in turn relies on the 2014 draft report from the WHO, the reports from the SSM and the 2015 report from the Scientific Committee on Emerging and Newly Identified Health Risk (SCENIHR).

There seems to be an "ICNIRP cartel" of experts that dominate different expert evaluations, *e.g.*, ICNIRP, WHO, SSM, SCENIHR. This seems also to be the case in this Health Council report, see Table 1.

As a general rule, scientists who are of the opposing opinion, *i.e.*, that there are health risks associated with exposure to RF radiation, have never been invited to take part of these expert groups. Thus, the opinions expressed in these reports are not representative of the opinions in the scientific community on effects from EMFs (https://www.emfscientist.org).

It is pertinent to evaluate the current ICNIRP 2020 conclusions on cancer risks from RF radiation because the Health Council of the Netherlands recommends using the ICNIRP 2020 guidelines. The recommendation to "take a cautious approach and keep exposures as low as reasonably achievable" is certainly difficult to achieve on a market with expanding wireless communication and may be of no practical use. It is assumed that the ICNIRP 2020 guidelines will be used in most countries. Thus, the influence by ICNIRP and its members on reports that form the basis for guidelines by different organizations is necessary to elucidate.

The ICNIRP

In the following, the evaluation of increased cancer risks according to ICNIRP 2020 is discussed in more detail. It should be mentioned that ICNIRP relies only on thermal (heating) effects from RF radiation. Nonthermal effects are dismissed thereby neglecting a large amount of scientific evidence on harmful nonthermal effects, for instance DNA damage[4], oxidative stress[5] and cancer[2]. It should be noted that the International Agency for Research on Cancer (IARC) at the WHO evaluation from 2011 is not included in the report. IARC concluded that RF radiation in the frequency range

Table 1 Members of World Health Organization monograph core group and their involvement in other groups, see Hardell[16], 2017

Name	WHO	ICNIRP	UK/AGNIR	SSM	SCENIHR
Simon Mann	X	X	X		
Maria Feychting	X	X	X	X[1]	
Gunnhild Oftedal	X	X			
Eric van Rongen	X	X		X	
Maria Rosaria Scarfi	X	X[1]		X	X
Denis Zmirou	X				

[1]Former. X: Describes that the person is a member of a specific group. WHO: World Health Organization; ICNIRP: International Commission on Non-Ionizing Radiation Protection; AGNIR: Advisory Group on Non-Ionizing Radiation; SSM: Strålsäkerhetsmyndigheten (Swedish Radiation Safety Authority); SCENIHR: Scientific Committee on Emerging and Newly Identified Health Risk.

30 kHz to 300 GHz is a possible human carcinogen, Group 2B after an evaluation by 30 international experts[6,7].

ICNIRP is a non-governmental organization based in Germany. It has obtained major influence worldwide on health risks from RF radiation through its recommended guidelines for limiting RF radiation exposure published in 1998, 2009 and 2020[8-10].

ICNIRP maintains the same attitude towards health effects from RF radiation as the IEEE and its standards setting Committee, the ICES. In fact, several members of ICNIRP are also members of these organizations, for instance van Rongen E, member of ICES since 2000. ICES is dominated by industry and military representatives[11]. ICES within IEEE also sets limits for RF exposure, which are in line with the ICNIRP's opinion that there are only immediate thermal effects, and no effects below those that cause immediate effects due to increased temperature.

As stated in an EU report[12], ICNIRP's chairman, van Rongen E, was invited in 2016 to the industry organization ICES to comment and thereby influence the upcoming ICNIRP 2020 guidelines. The report concluded that it is "clear from ICES minutes that ICNIRP worked very closely with IEEE/ICES on the creation of the new RF safety guidelines that were published in March 2020. And this implies that large telecom-companies such as Motorola and others, as well as United States military, had a direct influence on the ICNIRP guidelines, which are still the basis for EU- policies in this domain."

This adds to the evidence that van Rongen E, one of two secretaries of this Health Council, seems to have conflicts of interest.

In 2020, ICNIRP published new guidelines on health risks[10]. These updated guidelines were based on documents from the WHO, the SSM and the SCENIHR, as mentioned earlier, without any new thorough evaluation of its own.

Not one of these three reviews has been published after peer review in a scientific journal. In fact, substantial critique from the scientific community has been expressed against these reviews but has been ignored[13,14]. It should be noted that the most recent research is not covered in these older documents.

WHO

A draft of a monograph on health effects of EMF exposure was released by the WHO in 2014 but has never been published as a final version[15]. Public consultations were open until December 31, 2014.

It should be noted that the WHO in 2014 issued the following statement: "This is a draft document for public consultation. Please do not quote or cite"[15]. Nevertheless, this WHO draft from 2014, issued by a group dominated by ICNIRP members, among them van Rongen E, was used as a basis for the ICNIRP guidelines 2020 and this new report by the Health Council of the Netherlands. Five of six members of the WHO core group were members of ICNIRP, see Table 1[16]. The WHO group was nearly identical to ICNIRP.

The SCENIHR

The following quote is from the SCENIHR report 2015[17]: "Overall, the epidemiological studies on mobile phone RF EMF exposure do not show an increased risk of brain tumors. Furthermore, they do not indicate an increased risk for other cancers of

the head and neck region…The results of cohort and incidence time trend studies do not support an increased risk for glioma while the possibility of an association with acoustic neuroma remains open."

The SCENIHR report was criticized for its biased presentation and conclusions[13, 14]. Like the WHO draft report and the Swedish reports by SSM, it was written by a group of experts with no representation from the many scientists that report increasing evidence of harmful effects below the ICNIRP guidelines and demand better protection against health risks (https://www.emfscientist.org). SCENIHR included scientists with financial ties to industry or membership in ICNIRP or IEEE.

The SSM

Between 2003 and 2019, the SSM group has published reports in English on its webpage, three cited by ICNIRP 2020[18-20]. In line with ICNIRP, the SSM reports have ignored harmful effects from nonthermal RF exposure. Since the first report in 2003 until today, around half of the group's members have also been present or previous ICNIRP members. Both van Rongen E and Huss A have been part of this group of SSM experts. In consequence, the conclusions have generally been that there are no health risks below the limits recommended by themselves, *i.e.* in agreement with ICNIRP.

The 2018 annual report was the twelfth in this series and covered studies published from October 2015 up to and including March 2017. The conclusion was that "No new health risks have been identified"[20].

It should be noted that SSM in April 2020 published a new report from the SSM expert group that concluded: "The results of the research review give no reason to change any reference levels (ICNIRP's) or recommendations in the field." Of the ten members in the scientific group, five were present or past members of ICNIRP[21].

Current ICNIRP evaluation

van Rongen E, at that time chair of the ICNIRP Commission, claimed in a press release of the new ICNIRP 2020 guidelines that the 1998 version was "conservative in most cases" and "still provide adequate protection for current technologies." He also argued that "The most important thing for people to remember is that 5G technologies will not be able to cause harm when these new guidelines are adhered to"[22]. Because there is still no published peer-reviewed research showing no health effects from the new 5G technology, not even from short-term exposure, that is a statement without scientific foundation.

Many incorrect statements were made in the ICNIRP 2020 article[10]. In the following, the section on cancer is commented. That section starts at page 41 in the ICNIRP 2020 article with "There is a large body of literature concerning cellular and molecular processes that are of particular relevance to cancer. This includes studies of cell proliferation, differentiation and apoptosis-related processes, proto-oncogene expression, genotoxicity, increased oxidative stress, and DNA strand breaks. Although there are reports of effects of radiofrequency EMFs on a number of these endpoints, there is no substantiated evidence of health-relevant effects."

Regarding studies on cancer risks, no current evaluation is made. Several studies are not included, or references are not even given to the discussed studies. This is not easily understood by the reader that is not experienced in this area.

Regarding animal studies yielding a tumor promoting effect from RF radiation[23, 24], ICNIRP states that "interpretation of these results and their applicability to human health (is) difficult, and, therefore, there is a need for further research to better understand these results." In the next paragraph, the recent animal National Toxicology Program (NTP) studies[25,26] and Ramazzini Institute results[27] on animal carcinogenesis from RF radiation are disregarded stating that "no consistency was seen across these two studies" and "within the context of other animal and human carcinogenicity research (HCN 2014, 2016), their findings do not provide evidence that radiofrequency EMFs are carcinogenic."

That is a remarkable statement with no risk by ICNIRP and is not based on what the science really showed. There is a pattern of increased cancer risk based on human epidemiology, animal research and experimental findings. The Hardell team concluded that "There is clear evidence that RF radiation causes cancer/tumor at multiple sites, primarily in the brain (glioma) and head (acoustic neuroma). There is also evidence of an increased risk of developing other tumor types. The results are similar in both the NTP studies and the Ramazzini Institute findings (Falcioni *et al* [27]). Based on the IARC preamble to the monographs, RF radiation should be classified as Group 1: The agent is carcinogenic to humans"[28].

In a note published by ICNIRP, it was claimed that the histopathological evaluation in the NTP study was not blinded as to exposure status[29]. This false accusation was rebutted by one of those responsible for the NTP study[30]. However, the rebuttal seems to have had no impact on the current ICNIRP evaluation[10].

Regarding the 13 country Interphone case-control study on glioma[31] and acoustic neuroma[32], ICNIRP concluded that the studies do "not provide evidence of an increased risk," which is not correct. However, regarding glioma cumulative call-time of mobile phone ≥ 1640 h yielded odds ratio (OR) = 1.40, 95% confidence interval (CI) = 1.03-1.89. The risk increased over time for exposure and was highest in the most exposed parts of the brain. These findings were of biological relevance.

RF radiation dose was estimated in parts of Interphone as total cumulative specific energy (J/kg) absorbed at the tumor's estimated center taking into account multiple RF exposure determinants[33]. The risk increased with increasing total cumulative specific energy 7+ years before diagnosis, OR = 1.91, 95%CI = 1.05-3.47 (*P* value = 0.01) in the highest quintile. Compared with glioma in other parts of the brain increased, ORs were found for tumors in the most exposed part of the brain in those with 10+ years of mobile phone use, OR = 2.80, 95%CI = 1.13-6.94.

Grell *et al*[34] published similar results for the Interphone study. "We found a statistically significant association between the intracranial distribution of gliomas and the self-reported location of the phone…Taken together, our results suggest that ever using a mobile phone regularly is associated with glioma localization in the sense that more gliomas occurred closer to the ear on the side of the head where the mobile phone was reported to have been used the most."

For glioma, when comparing those in the highest quartile of use (> 558 lifetime hours) to those who were not regular users, the OR was 2.0, 95%CI = 1.2-3.4 in the Canadian part of Interphone[35]. After adjustment for selection and recall biases, a somewhat higher OR was found, 2.2, 95%CI = 1.3-4.1.

The Interphone study gave for acoustic neuroma statistically significant increased risk. Thus, ipsilateral cumulative mobile phone use > 1640 h gave OR = 2.33, 95%CI = 1.23-4.40[32].

Regarding the Hardell team studies, ICNIRP writes: "a set of case-control studies from the Hardell group in Sweden report significantly increased risks of both acoustic neuroma and malignant brain tumors already after less than five years since the start of mobile phone use, and at quite low levels of cumulative call time." The studies are not carefully evaluated by ICNIRP and no references are given. On the contrary, overall there was no statistically increased risk in the shortest latency period > 1-5 years for glioma or acoustic neuroma[36,37]. Somewhat higher risk was seen for ipsilateral exposure; a promotor effect cannot be excluded in addition to initiation of cancer[36]. Also in the Hardell team studies the risk increased with latency and cumulative use and was highest in the most exposed areas of the brain that would be expected for carcinogenesis. Concepts of both promotion, initiation and biological relevance for RF radiation carcinogenesis must be considered when discussing results in different studies. That is obviously not the case for ICNIRP.

In addition, ICNIRP claims that the Hardell team results may be caused by recall bias. For meningioma, no statistically significant increased risk was found in the same study. Using meningioma cases as "controls" (the comparison entity) still yielded statistically significant increased risk for glioma and mobile phone use[36]. Similar results were found for acoustic neuroma using meningioma cases as the comparison group[37]. These results clearly show that the increased risks for glioma and acoustic neuroma were not caused by recall bias, *i.e.* cases tending to overestimate exposure. That would have applied to all cases regardless of tumor type.

ICNIRP omitted the CERENAT study by Coureau *et al*[38]. The study strengthened the evidence of increased risk for glioma associated with mobile phone use. Life-long cumulative duration ≥ 896 h gave OR = 2.89, 95%CI = 1.41-5.93 for glioma. Number of calls ≥ 18360 gave OR = 2.10, 95%CI = 1.03-4.31. Higher risks were obtained for the highest exposed area, the temporal brain lobe, as well as occupational and urban mobile phone use.

In spite of serious methodological limitations, the Danish cohort study on mobile phone use was included, adding to the ICNIRP no-risk conclusion. This study, partly funded by Danish telecom operators, first published in 2001[39] and updated in 2011 [40], reported no increased risks of tumors in the central nervous system. It was based on 420095 mobile phone private subscribers. This group's incidence of brain tumors was compared to the incidence within the rest of the Danish population (control group). However, there are several methodological limits in the study such as inclusion only of mobile phone private subscribers in Denmark between 1982 and 1995 in the exposure group. The most exposed group, corporate users, and subscription in

1996 or later were excluded. Use of cordless/DECT phones was disregarded although shown to be a risk factor for brain and head tumors[36,37].

The study has been called "The most severely biased study among all studies published so far"[41]. Certainly, there were severe methodological flaws. The Danish cohort study was regarded by IARC in the 2011 evaluation[7] to be uninformative regarding cancer risks due to serious exposure misclassification. However, it was included by SCENIHR[17], WHO[15], SSM (2013)[42], and ICNIRP[10] as evidence of no risk. The statement by SSM 2013[42] that "The Danish cohort studies make an important contribution to the total assessment in the field" is not correct. The shortcomings in the study should have been known to the SSM expert panel as discussed in a peer-reviewed article[43] concluding that "After reviewing the four publications on the Danish cohort study, one might rightly wonder whether this cohort was initially set up to show no increased risk."

The ICNIRP 2020[10] reference levels for RF radiation are based on time averaged exposure over 6 min or 30 min. However, pulses from different RF radiation sources may give much higher peak radiation from short time pulses than the power density average. Using time averaging in reference values may thus underestimate the risk. In addition, ICNIRP 2020 suggests higher guidelines for local exposure. According to the article, the reference level, *e.g.*, > 2 to 6 GHz local exposure, is suggested to increase to 40 W/m^2 time averaged over 6 min. This is contrary to the increasing scientific evidence on detrimental effects on human health and the environment from RF radiation. Previously 10 W/m^2 was used as reference level time averaged 6 min exposure[8,9]. Now the reference level for whole body exposure, time averaged 30 min, is suggested to be 10 W/m^2.

The evidence for cancer

Several meta-analyses have during the last years reached the conclusions that all together the available evidence shows increased risk of cancer from mobile phone use [2]. One additional method is to use Sir Bradford Hill's viewpoints from 1965 on association or causation written at the height of the tobacco and lung cancer controversy[44]. In an article published in 2017, these viewpoints were used to evaluate RF radiation carcinogenesis based on epidemiology and laboratory studies [45]. It was concluded that based on these Hill "criteria" RF radiation should be regarded as a human carcinogen causing glioma. Since then the evidence has strengthened.

As discussed above, studies have shown an association between glioma and acoustic neuroma and mobile phone use especially in: (1) people with longest latency (time from first use until tumor diagnosis); (2) people with highest cumulative use of mobile phones; (3) people who had used mobile phones on the same side of the head as that on which their tumor developed; and (4) in people whose tumor was in the temporal lobe of the brain, *i.e.* the highest exposed lobe during use of the handheld wireless phone.

In addition, the Hardell team studies from Sweden found similar results for use of cordless phones. Thus, based on these findings that are of biological relevance and supported by the Hill viewpoints, a causal interpretation is possible. Thus, RF radiation should be classified as a human carcinogen, Group 1 according to the IARC criteria[45,46]. However, only IARC can make that classification.

DISCUSSION

In order to achieve sustainable development, policies must be based on the precautionary principle. No doubt there are threats of serious or irreversible damage by exposure to RF radiation, not the least the increased risk for glioblastoma with short survival for those affected[47]. Lack of full scientific certainty, as proposed by certain public health organizations, should not be used as a reason for postponing measures to prevent environmental degradation. Thus, a moratorium on the deployment of 5G and considerable reduction of RF radiation from existing systems is urgently needed. In short, "The precautionary principle provides justification for public policy actions in situations of scientific complexity, uncertainty and ignorance, where there may be a need to act in order to avoid, or reduce, potentially serious or irreversible threats to health or the environment, using appropriate strengths of scientific evidence, and taking into account the likely pros and cons of proportionate actions and inactions" [48].

In contrast to that as a general rule, ICNIRP, WHO, SSM, and SCENIHR have for many years dismissed available studies showing harmful effects from nonthermal RF radiation exposure and have based their conclusions mainly on studies showing no effects. Thereby results showing health hazards are criticized or not even cited in contrast to studies showing no risks that are accepted as evidence of no risk in spite of severe methodological problems. Many of the statements by these agencies are misleading and not correct. They are easily rebutted by reading the relevant publications. In fact, an Italian court ruling linked mobile phone use to tumors already in 2012. Also, later court rulings in Italy have come to the same conclusion.

These ICNIRP cartel dominated expert groups consequently reach similar conclusions that there are no health effects below ICNIRP guidelines. Scientists with opinions that there is increasing evidence of health risks below the ICNIRP guidelines, *e.g.*, as expressed in the EMF Scientists Appeal, are not invited to expert groups at the WHO, the EU (SCENIHR), the SSM, or ICNIRP. Table 1 clearly illustrates that few persons constitute different groups aimed at preventing hazards and risks to the health and the environment. The ICNIRP view is thereby influencing these expert reports, which also formed the basis for this Health Council report in the Netherlands.

There is growing scientific evidence on health risks from the RF radiation emitted by existing telecommunications systems prior to 5G. In addition, 5G will lead to an increase of radiation, including new techniques, which leads to the conclusion that hazardous exposure will increase. However, it is unacceptable that there is scanty research being performed on the risks or hazards from the new 5G technology and the new frequencies that will be used. This means an experiment on human health and the environment that should not be accepted. Thus informed consent would be needed from each involuntary exposed person. The exposure guidelines in ICNIRP 2020[10] constitute a hazard to public health and the environment because evidence is abundant on harmful effects including DNA damage, oxidative stress, and cancer.

The Health Council Committee argues that "epidemiological research into the relation between exposure to the 5G frequencies used and the incidence of cancer, reduced male fertility, poor pregnancy outcomes and birth defects" should be performed after the roll-out of the 5G and its increase in radiation. However, the same Committee in the same report claims that "The committee has however classified relations as "possible" for cancer, male fertility, pregnancy outcomes and birth defects."

Further the Committee states that "The conclusions from those earlier reports are also relevant for 5G as far as the frequency bands around 700, 1400, 1800 and 2100 MHz are concerned. The committee sees no reason to limit or stop the use of the lower frequency bands for 5G. Frequencies near 700 MHz and 3.5 GHz have been in use for current telecommunication systems or other applications such as Wi-Fi for years, without any demonstrable health damage as a result."

Thus, this report states that 5G at the lower frequencies has no "demonstrable health damage, although relations (are), "possible" for cancer, male fertility, pregnancy outcomes and birth defects".

The least to say is that the Committee is not transparent regarding health risks; they are "possible" but for the roll-out of 5G they are not "demonstrable." Research on health hazards must be done before exposure, not after, something that is suggested for the higher frequencies. "The committee recommends not to use the 26 GHz frequency band for 5G for as long as the potential health risks have not been investigated."

For implementation of 5G, regardless of frequency, ethics in medicine should be applied. In medicine the patient must be informed about the risks but also benefits in experimental studies and give written consent for the participation. That should also apply to the deployment of 5G. However, it has not been done so far. The participation is forced upon everybody, which is of course unacceptable from a human rights perspective.

CONCLUSION

In conclusion regarding cancer, current scientific evidence clearly demonstrates an increased risk for glioma and acoustic neuroma for use of mobile and/or cordless phones. In this review other tumor types and health endpoints are not discussed. The increased risk for brain and head tumors is based on human cancer epidemiology studies and is supported by similar tumor types found in animal studies. In fact, these animal studies confirmed the earlier results in case-control studies on increased tumor

risk for use of wireless phones (both mobile and cordless phones). Mechanistic aspects on carcinogenesis come from laboratory findings on, *e.g.*, the increase of reactive oxygen species[5] and DNA damage[4].

The current evaluation by the Health Council of the Netherlands is based on a WHO draft and SSM report. It also recommends using ICNIRP guidelines, considered to be insufficient to protect against health hazards, such as cancer, by the majority of the scientists in this field (https://www.emfscientist.org). The report does not represent a thorough, balanced, objective, and up-to-date evaluation of cancer risks and other hazardous effects from RF radiation. It is also strikingly contradictory as it concludes that serious health effects such as cancer and birth defects are "possible." Yet it has no objection to the roll-out of 5G and recommends that later studies are performed to study health outcomes such as cancer and birth defects. Thus, no lessons are learned from existing observations on increased cancer risks[49].

The conclusion by the Commission that there is no reason to stop the use of lower frequencies for 5G up to 3.5 GHz because of no "proven adverse health effects," merely reflects the biased conclusions by ICNIRP dominated groups. Thus that conclusion must be dismissed, and new guidelines for previous and new frequencies must be established considering the new technology, the different propagation pattern for 5G, and increased RF radiation.

A moratorium is urgently required on the implementation of 5G for wireless communication[13]. Ultimately, wired solutions are preferred.

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REVIEW

Breast cancer: Muscarinic receptors as new targets for tumor therapy

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Abstract

The development of breast cancer is a complex process that involves the participation of different factors. Several authors have demonstrated the overexpression of muscarinic acetylcholine receptors (mAChRs) in different tumor tissues and their role in the modulation of tumor biology, positioning them as therapeutic targets in cancer. The conventional treatment for breast cancer involves surgery, radiotherapy, and/or chemotherapy. The latter presents disadvantages such as limited specificity, the appearance of resistance to treatment and other side effects. To prevent these side effects, several schedules of drug administration, like metronomic therapy, have been developed. Metronomic therapy is a type of chemotherapy in which one or more drugs are administered at low concentrations repetitively. Recently, two chemotherapeutic agents usually used to treat breast cancer have been considered able to activate mAChRs. The combination of low concentrations of these chemotherapeutic agents with muscarinic agonists could be a useful option to be applied in breast cancer treatment, since this combination not only reduces tumor cell survival without affecting normal cells, but also decreases pathological neo-angiogenesis, the expression of drug extrusion proteins and the cancer stem cell fraction. In this review, we focus on the previous evidences that have positioned mAChRs as relevant therapeutic targets in breast cancer and analyze the effects of administering muscarinic agonists in combination with conventional chemotherapeutic agents in a metronomic schedule.

Key Words: Muscarinic receptors; Drug therapy; Breast cancer; Drug combination; Metronomic therapy; Drug resistance

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Core Tip: Muscarinic acetylcholine receptors should be considered as new targets in breast cancer therapy since they are expressed in breast tumor tissue but not in normal tissue. The addition of muscarinic agonists at low concentrations combined with traditional chemotherapeutic drugs presents promising results. The administration of these combinations in a metronomic schedule is effective to kill tumor cells, reduce tumor invasion and neoangiogenesis. Promising results have also been reported regarding the expression of drug extrusion pumps and the decrease of the cancer stem cell fraction, which would be useful to reduce resistance to traditional chemotherapy and the relapse of breast cancer.

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INTRODUCTION

Cancer is a heterogeneous disease characterized by the loss of normal behavior of cells and the acquisition of new characteristics that lead to malignant transformation. These characteristics include high growth and division rate, and the ability to invade neighboring tissues and to disseminate to distant organs to generate metastases[1].

For many years, researchers thought that tumors had a clonal origin[2,3], but taking into account the high intra-tumor heterogeneity observed, they later considered the co-existence of different cell subpopulations within a tumor. One of these subpopulations, known as cancer stem cells, was identified in 1994 in an acute myoleid lymphoma[4] and later described in several solid tumors such as lung, breast, colon, prostate and brain tumors[5-8]. These cells have self-renewal and differentiation capacity, and also exhibit high expression of drug extrusion pumps, the latter of which confers them resistance to chemotherapy[9]. In addition, several authors have described that this cell population is responsible for the failure of response to cancer treatment and for cancer recurrence[10-12].

Also, it has been demonstrated that primary and metastatic tumor cells present phenotypic, genotypic and epi-genotypic differences[13]. These differences result in several changes in the expression and function of membrane protein receptors as well as in their signaling pathways. Thus, this intra-tumor heterogeneity creates a great challenge in the selection of specific biomarkers and treatments in oncology[14].

The transformation of a normal cell into a tumor one is a complex and progressive process in which the cell acquires genetic modifications like deletions or other mutations in tumor suppressor genes and/or oncogenic genes. Since these genes control cell proliferation either directly or indirectly, deletions or mutations in these genes may allow tumor cells to grow without control, disseminate and invade other tissues[15].

Tumor suppressor genes can either inhibit the cell cycle or promote apoptosis. The loss of their functionality can cause the development of cancer, as demonstrated in ovarian[16], lung[17], colorectal[18], head and neck[19], pancreatic[16], uterine[20], osteosarcoma[21], gastrointestinal[22], bladder[23] and breast[24] tumors.

The presence of several of these genes is associated with a high incidence of cancer. These genes can be divided into highly and moderately penetrant in relation to cancer. Highly penetrant genes include *BRCA1*, *BRCA2*, *TP53*, *PTEN*, *CDH1*, *STK11* and *PALB2*, whereas moderately penetrant ones include *CHEK2*, ATM, *BARD1*, *BRIP1*, *NBN*, *NF1*, *RAD51D* and *MSH6*[25-27].

Oncogenes, on the other hand, are abnormal or mutant genes related to normal genes called proto-oncogenes, which support tumor development. From the beginning of the 1980's, the participation of these genes has been studied and associated with tumor progression[28]. Many studies have demonstrated that proto-oncogenes affect the activity of telomerase enzyme in different tumor cell types. Due to the latter, these cells exhibit short telomeres and chromosomal instability[29]. Somatic mutations in the telomerase gene promoter have been described in gliomas[30], head and neck cancer [31], thyroid carcinoma[32], hepatocellular carcinoma[33], squamous cell carcinoma [34], bladder cancer[35], breast carcinoma[36] and melanomas[37].

In an incipient stage of tumor growth, when malignant cells exert a rapid rate of proliferation, the nutritional requirements of cells increase, leading to an angiogenic switch. The latter implies the development of new blood vessels from the ones preexisting in the tumor environment, which allows the emerging tumor to duplicate its diameter from 1 mm to 2 mm[38]. Concomitantly, changes in the cellular biological properties lead tumor cells to invade the surrounding extracellular matrix, to intravasate into the nearby blood and lymphatic vessels, and then to disseminate through the circulation to distant organs and to metastasize[39].

According to data from the International Agency for Research on Cancer, in 2020 this illness produced more than 19 million new cases and caused almost 10 million deaths worldwide. In particular, breast cancer has the highest incidence among all female cancer types. It represents 24.5% of the total new recorded female cancer cases and is one of the main causes of death in women, corresponding to 15.5% of deaths from this disease (Figure 1)[40].

Since life expectancy in the world population has become higher, the risk of developing tumors has also increased, considering that this parameter increases with age[41]. It is expected that the number of new cases of breast cancer in 2040 will rise to 3.2 million people and there will be more than 1 million deaths unless treatments present higher effectiveness[40].

BREAST CANCER

The breast is composed of lobules (which are milk-producing glands), the ducts that connect the lobules to the nipple, and connective, fatty and lymphatic tissues. Breast cancer occurs when there is an uncontrolled growth of cells within any of these components. Although tumors can appear in any tissue of the breast, it occurs most frequently in the lobules[42].

Breast tumors exhibit particular histopathological and biological characteristics that require specific and different antitumor strategies. For these reasons, the adequate classification of tumors has a main therapeutic importance[43-45].

The different types of therapies against breast cancer include surgery, radiation and the administration of immunobiological or chemotherapeutic agents[46,47].

Surgery (tumorectomy or mastectomy) is one of the main options of treatment for patients at different stages of this disease, whereas radiotherapy is used as a complement to surgery and/or chemotherapy to reduce the probability of tumor relapse. The combination of tumorectomy and radiotherapy is used as a replacement for mastectomy in patients at the first stages of the disease.

Chemotherapy consists of the use of drugs to kill tumor cells. It can be administered as adjuvant therapy, when drugs are applied after another treatment like surgery in order to eradicate tumor cells that might have survived. Oncologists also administer neoadjuvant chemotherapy before surgery to reduce the tumor size and to be more likely to completely eliminate the tumor after the procedure[40].

As described below, according to their mechanism of action, chemotherapeutic agents can be classified into: endocrine drugs, immunological agents, DNA alkylating agents, antimetabolites and antimitotic drugs[48].

Endocrine drugs are used to treat estrogen receptor (ER) or progesterone receptor (PR) positive breast tumors. These drugs are synthetic analogs of the anti-gonadotropin-releasing hormone, anti-progestins or anti-estrogens. The latter can be divided into aromatase inhibitors and ER antagonists. The most common side effects of these drugs are: flushing sensation, nightly sweats, vaginal dryness, high blood clot risk, apoplexy, cataracts, endometrial cancer, uterus cancer, bone-mass decrease and gastrointestinal symptoms[40,49].

Immunological agents are drugs that can stimulate the patient´s immune system to detect and eliminate breast cancer cells. Immunological agents also include monoclonal antibodies against specific tumor cell proteins, and cancer treatment vaccines. Different types of immunological agents can be administered to inhibit check-points, block the suppression of the immune response and/or over-activate T-cells from patients. The most common side effects of these drugs are: fatigue, fever, shivers, weakness, nausea, vomits, dizziness, body aches and high or low blood pressure[40,50-52].

Regarding DNA alkylating agents, the ones used to treat breast cancer include cyclophosphamide and doxorubicin. Cyclophosphamide, which is an oxazaphosphorine, interferes with the duplication of DNA and RNA transcription, and its most common undesired effects are: myelosuppression, hepatotoxicity, pulmonary fibrosis,

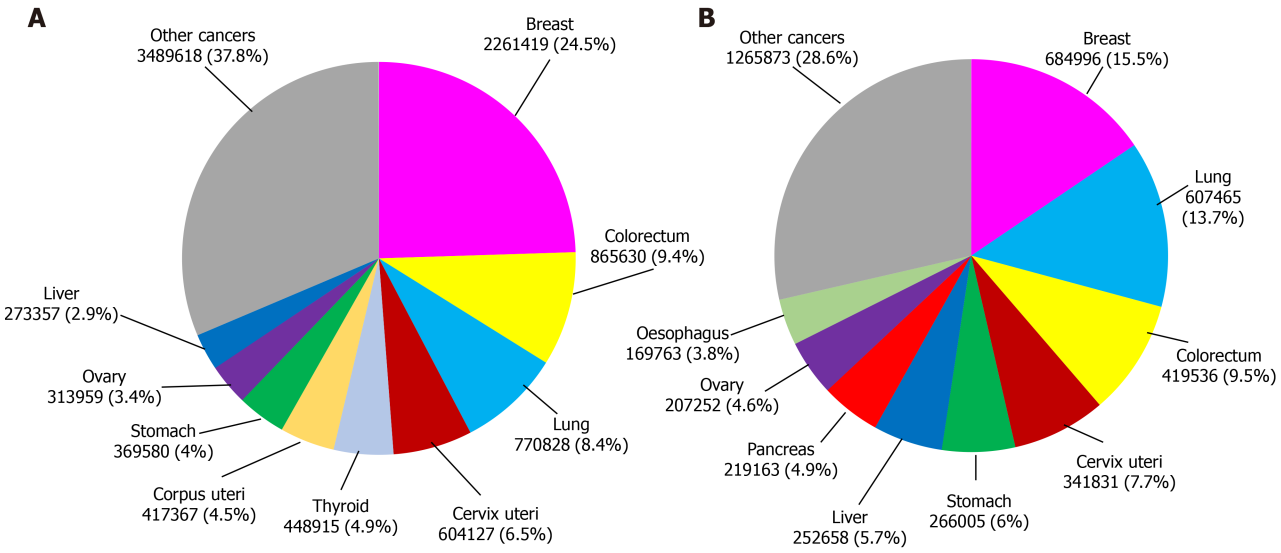


Figure 1 Female world incidence of new cases of different types of cancer and number of cancer deaths in 2020. A: Different types of cancer; B: Number of cancer deaths.

nephrotoxicity, mucosis, megaloblastic anemia, birth defects and neurotoxicity[40,53].

Doxorubicin, which is an anthracycline, is a topoisomerase II inhibitor that increases DNA degradation, preventing cell replication. Additionally, it can intercalate into DNA and promote the formation of free radicals, which in turn causes the fragmentation of DNA strands. Unfortunately, this agent can also cause cardiotoxicity, myelosuppression, alopecia, extravasation, infertility and urine discoloration[40,53].

The antimetabolites used in breast cancer can be divided into antifolates (such as methotrexate) and pyrimidine antagonists (such as 5-FU, capecitabine or gemcitabine). Methotrexate is a competitive inhibitor of the dihydrofolate reductase which causes a decrease in pyrimidine and DNA synthesis. On the other hand, 5-FU or capecitabine can form a complex with the thymidylate synthase-folic acid, inhibiting its activity and reducing thymidine and DNA synthesis, whereas gemcitabine can incorporate a pyrimidine analog into DNA, decreasing its synthesis. The most common collateral effects of these drugs are: myelosuppression, cardiotoxicity, hepatotoxicity, pulmonary toxicity, hemolytic uremic syndrome and hyperammonemic encephalopathy[40,53].

Finally, the antimitotic drugs used in breast cancer treatment can be divided into three types: vinca alkaloids (such as vinorelbine), taxanes (such as paclitaxel and docetaxel) and non-taxane microtubule inhibitors (such as eribulin, ixabepilone and epothilone). Vinorelbine binds to B tubulin, inhibiting its polymerization into microtubules; it also prevents the formation of the mitotic spindle and arrests cells in the M phase. Taxanes produce hyper-stabilization in polymerized microtubules, thus inhibiting the degradation of the mitotic spindle, and also arrest cells in the M phase. Non-taxane microtubule inhibitors can either prevent the formation of the mitotic spindle (eribulin) or bind to B tubulin, hyper-stabilizing microtubules and producing cell arrest. Undesirable actions of these drugs are: myelosuppression, peripheral neuropathy, QT prolongation and hypersensitivity[40,54-56].

Selecting the most effective chemotherapeutic agent to treat breast tumors requires the determination of their genetic profile. Tumors are classified according to the immunohistochemical analysis of the protein expression levels of ER, PR, human epidermal growth factor receptor type 2 (HER2) and Ki-67. This analysis allows defining four breast cancer subtypes: (1) Luminal A; (2) Luminal B; (3) HER2 positive; and (4) Basal-like or triple negative[57,58].

Luminal A tumors express ER and/or PR, are HER2 negative and present low levels of Ki-67 protein, which modulates tumor cell growth[59]. This tumor subtype is the most frequently diagnosed annually and has a 5-year relative survival rate of 94%[60]. The conventional treatment for these tumors is endocrine therapy[61].

Luminal B tumors express ER and/or PR and can be positive or negative for HER2 expression. This tumor subtype has a higher cell proliferation rate than the luminal A subtype because it expresses a higher concentration of Ki-67 protein[61]. Luminal B tumors represent 10% of the total of annually diagnosed breast tumors and have a 5-year relative survival rate of 90%[60]. This tumor subtype is usually treated with endocrine therapy and in some cases with HER2 targeting drugs or other chemothera-

peutic agents[61,62].

HER2 positive tumors have a high expression of this molecule and do not express ER or PR. These tumors also have a high concentration of the Ki-67 protein[59]. This tumor subtype represents 5% of all annually diagnosed breast tumors and has a 5-year relative survival rate of approximately 80%[60]. HER2 positive tumors are usually treated with HER2 targeting antibodies like trastuzumab[61] or margetuximab[63,64] alone or combined with other chemotherapeutic agents[65,66].

Triple negative tumors do not express ER, PR or HER2, but express a high concentration of Ki-67 protein and are usually very invasive and agressive[59]. This tumor subtype represents 10% of all annually diagnosed breast tumors and has a 5-year relative survival of approximately 80%[60]. These tumors can be subdivided into basal, claudin-low, normal-like and other less frequent subtypes[43,67], and, since they do not have a specific therapeutic target, they do not have any specific treatment. Although some authors indicate the use of a platinum-based agent such as cisplatin, the results are not encouraging due to the low life expectancy of the patients[68,69].

The 5-year relative survival rate varies significantly from patients with luminal A tumors, who have the best prognosis, to patients with triple negative tumors, who have the worst prognosis. The decrease in the survival rate observed in triple negative tumor patients can be explained by different factors, including higher resistance to chemotherapy and/or radiotherapy, higher relapse probability, and higher ability to metastasize in comparison with other tumor subtypes[70].

Although in recent years the diagnosis, classification and treatment of breast cancer have improved, treatment failure, recurrence and mortality are still reported worldwide.

It must be taken into account that, one of five women worldwide will develop cancer during her lifetime and one of eleven will die from this illness[40].

MUSCARINIC ACETYLCHOLINE RECEPTORS

Muscarinic acetylcholine receptors (mAChRs) are G protein-coupled receptors (GPCRs), which belong to the superfamily of seven-transmembrane domain receptors and can modulate many functions in normal and tumor cell biology[71,72]. Regarding the latter, several authors have described an increase in the expression of different GPCRs in tumor tissues. GPCRs include thrombin receptor[73], protease-activated receptor-1[74], angiotensin II receptor type I[75], GPR161[76], GPR81[77] and leucine-rich repeat-containing G-protein-coupled receptor 5[78].

mAChRs were firstly described in the central nervous system[79] and then in the parasympathetic nervous system[80]. More recently, mAChRs, together with nicotinic receptors, have also been localized in non-neuronal cells. Also, their ligand, acetylcholine (ACh) and the enzymes that synthesize and degrade it have also been detected out of the nervous system, defining a new organization known as the non-neuronal cholinergic system[81]. In our laboratory, we described for the first time the over-expression of mAChRs of the GPCR family in breast tumor tissues and in cancer cell lines from murine and human origin[82,83].

mAChRs are metabotropic receptors and five subtypes (M1 to M5) have been identified. When activated, they can trigger different signaling pathways known as canonical or non-canonical in distinct tissues[84].

These receptors are glycoproteins with seven hydrophobic transmembrane domains connected by three extracellular and three intracellular hydrophilic loops. The domains assemble forming a structure with a pocket where the agonist binds. Also, the cytoplasmic region of the receptor couples to G protein, which is composed of three subunits: α, β and γ[85]. When a ligand binds to the receptor, guanosine diphosphate is released and replaced by guanosine triphosphate, while the subunits are dissociated into a βγ dimer and the guanosine triphosphate-bound α monomer. Depending on the α subunit type (Gαs, Gαi, Gαq, and Gα11) different downstream effectors are stimulated[86].

Agonists like ACh or the synthetic non-hydrolyzable analog carbachol can activate M1, M3 and M5 receptors, which couple to a Gαq protein, which in turn up-regulates phospholipase C activity. This enzyme cleaves phosphatidylinositol 4,5-bisphosphate into 1,2-diacylglycerol and inositol 1,4,5-triphosphate. 1,2-diacylglycerol activates protein kinase C, which stimulates downstream proteins, causing calcium influx. Inositol 1,4,5-triphosphate leads the sarcoplasmic reticulum to release stored calcium, which modulates the activation of many calcium-dependent enzymes like nitric oxide synthase[87]. Additionally, the M3 receptor can activate Ras-Raf-1-Erk-Akt through a

non-canonical pathway[88].

In turn, the activation of M2 and M4 receptors, which are coupled to a Gαi/o protein, inhibits adenylyl cyclase, decreasing cyclic adenosine 3',5'-monophosphate (cAMP) production from ATP. The decrease in cAMP concentration subsequently blocks the activation of protein kinase A[87]. M2 and M4 receptors can also regulate the activity of potassium and calcium channels[89].

The expression of the different mAChR subtypes changes throughout different body organs and tissues. This differential expression leads to diverse responses to the same stimuli. Therefore, the identification of the mAChR subtypes in different cell types is important, especially because they could have therapeutic potential. The nervous system, for example, expresses all subtypes[90-92], whereas the M1 receptor is mainly expressed in salivary glands[93], pancreas[94], bladder[95] and respiratory pathways [96]. The M2 receptor subtype is predominantly expressed in cardiac[97], digestive[98] and respiratory[99] tissues, whereas the presence of M3 receptor protein has been documented in salivary glands[93], pancreas[100], bladder[101], lung[102], colon[103] and gastric smooth muscle[104]. Finally, the M4 and M5 receptors are also expressed in the lung[105,106], while the M4 subtype is also detected in gastric tissue[107].

The expression of mAChRs in different tissues can be regulated by various stimuli. Grodzki *et al*[108] demonstrated that the expression of M2 receptor can be increased by the treatment of sympathetic neurons with gamma interferon. In line with these results, at our lab, we demonstrated that the *de novo* expression of M3 and M5 receptors can be induced by the treatment of NIH3T3 fibroblasts with interferon gamma plus lipopolysaccharide from *Escherichia coli*, and that this leads to an increase in the sensitivity of these cells to carbachol[109].

In several diseases, including cancer, mAChRs or their signaling pathways are differentially expressed in comparison to healthy tissues. This could be useful at the moment of considering these receptors as therapeutic targets, as described in many respiratory diseases[110]. It has been demonstrated that in pulmonary arterial hypertension, for example, the administration of M3 receptor agonists induces an anti-hypertensive therapeutic response[111]. Additionally, in chronic obstructive pulmonary disease, it has been described that the usage of muscarinic antagonists combined with beta2 adrenergic receptors reduces hyperinflation, improves dyspnea, and reduces exacerbations while improving cardiac functions[112]. This combination of muscarinic antagonists and beta2 adrenergic receptors has also been studied as a therapeutic treatment for asthma, showing promising results[113].

Alterations in the expression of mAChRs or their signaling pathways have also been detected in several diseases of the central nervous system[114-117]. However, researchers could not determine whether these alterations are either the causes or the consequences of these pathologies[118]. In spite of this, muscarinic therapy has shown promising results for these diseases. It has been documented that the treatment of patients with Alzheimer or Huntington diseases with M1 receptor agonists causes beneficial effects, reducing the symptoms of these diseases[119,120]. Furthermore, the usage of M1 antagonists in Parkinson's disease and multiple sclerosis leads to positive results, also reducing the disease symptoms[121,122].

Different authors have determined that the expression of mAChRs in malignant tissues is different from that in normal tissues. These differences comprise an increase in the expression of receptors and/or a modulation in the subtype expression pattern. In human colon cancer, for example, the expression of the M3 receptor subtype is increased more than 100-fold respect to normal tissue and its activation modulates cell proliferation, progression and invasion of this neoplasia[123]. Also, in small cell lung cancer, the M3 receptor subtype is up-regulated, promoting cell migration and invasion[124]. In human bladder, while normal tissue expresses M1, M2 and M3 receptors, tumor tissue expresses only the M2 receptor and its activation induces a decrease in cell proliferation and migration[125]. Regarding breast cancer, several studies from our laboratory have demonstrated the presence of different mAChR subtypes in breast cancer cells of murine and human origin that promote tumor growth and angiogenesis, and the absence of these receptors in normal mammary cells [126-129].

The predominant expression of different mAChR subtypes in human tumors is summarized in Table 1.

It has been reported that the activation of mAChRs leads to the stimulation of several steps of tumor progression, involving different receptor subtypes in each tumor. Cell proliferation has been found to be induced by the M5 receptor subtype in melanoma cells[130], by the M1 and M4 receptor subtypes in glioblastoma cells[131], by the M3 receptor subtype in stomach and colon cancer[132,133], by the M2 receptor subtype in non-small cell lung cancer[134] and by all receptor subtypes in the

Table 1 Summary of the expression of muscarinic acetylcholine receptor subtypes (M) in different human cancers

Receptor subtypes	Malignant tissue	
M1	Skin[142]	Breast[128]
	Brain[131]	Liver[140]
	Prostate[136]	Esophagus[135]
	Cervix uteri[139]	Pancreas[147]
M2	Lung[134]	Esophagus[135]
	Brain[143]	Bladder[125]
	Breast [128]	Leukemia[145]
M3	Lung [124]	Breast[148]
	Colon[133]	Esophagus[135]
	Skin[142]	Liver[139]
	Prostate[138]	Stomach[132]
	Cervix uteri[139]	Leukemia[145]
	Head and neck[146]	
M4	Brain[131]	Breast[128]
	Prostate[137]	Esophagus[135]
	Cervix uteri[139]	Oral cavity[141]
M5	Skin[130]	Breast[128]
	Cervix uteri[139]	Esophagus[135]

esophagus[135].

In other types of tumor, researchers have observed not only an increase in cell proliferation, but also an increase in cell invasiveness and migration ability. This increase is induced by the M1, M3, M4 receptor subtypes in prostate tumors, by the M1, M3, M4 and M5 receptor subtypes in cervical tumors[136-139] and by the M1 and M3 receptor subtypes in hepatocellular carcinoma[124,140]. Finally, in oral cavity tumors, cell motility and dissemination have been found to be increased by the activation of the M4 receptor subtype[141].

Another important aspect to analyze is the expression and function of mAChRs in primary tumors and their metastases. Regarding the latter, two melanoma cell lines and a third one derived from a metastasis have been found to express M1 and M3 receptors. Although all of them responded to carbamylcholine, increasing cytosolic calcium levels, the metastatic cell line responded with a higher peak in calcium concentration. The authors speculated that this difference could be responsible for a higher malignance and migratory potential in metastasis[142]. In accordance with the latter observations, we have reported that the expression of mAChRs is significantly higher in the metastatic murine mammary cell line LMM3 than in the non-metastatic LM3 tumor cells that originated them[82]. However, when analyzing the function of mAChRs in tumor tissues, other authors confirmed opposite results, particularly considering the expression and function of the M2 receptor subtype. Lucianò *et al* [143], for example, established that two cell lines derived from a neuroblastoma with bad prognosis, SK-N-BE and SK-N-BE(2C), express the M2 receptor and that its activation inhibits the cell cycle. In human bladder tumors, Pacini *et al*[125] reported the expression of M2 receptor protein and found that its stimulation reduced cell proliferation and migration. These results are in line with the findings of Alessandrini *et al*[144], who demonstrated that the activation of the M2 receptor increases apoptosis in glioblastoma, a brain tumor with bad prognosis. Regarding leukemia, Cabadak *et al* [145] identified the presence of the M2 and M3 receptors in K562 cells, and linked their activation to an inhibition in cell proliferation. In head and neck cancer, Sun *et al*[146] reported a similar effect by M3 activation. Finally, in human pancreas tumor, Renz *et al* [147] demonstrated that M1 receptor activation decreases tumorigenesis.

Regarding breast cancer, we have reported the expression of all mAChR subtypes in the breast adenocarcinoma cell lines LM2 and LM3 derived from spontaneously aroused tumors in female BALB/c mice and the lack of expression of these receptors in

the murine mammary non-tumorigenic cell line NMuMG[82]. Using tritiated quinuclidinyl benzilate ([3H]-QNB) in binding assays, we determined the expression mainly of the M2 and M3 receptor subtypes[126]. We also found that the addition of carbachol to tumor cells induces two opposite actions depending on the concentration of the agonist and the time of treatment. At low concentrations added for 1 h or less, we reported a stimulation in cell proliferation, migration and also in tumor angiogenesis, whereas at higher concentrations or longer periods of treatment, we observed cell death[126]. These differences due to distinct experimental conditions besides the expression of different mAChRs could explain the opposite results documented in the literature previously mentioned.

Luminal breast cancer tumors are characterized by the expression of their endocrine receptor. In MCF-7 cells, the human luminal breast cancer cell line most studied in oncology research, we identified the expression of the M3 and M4 receptors by Western blot. We also found that the short-time treatment of MCF-7 cells with carbachol activates, through the M3 receptor, a phospholipase C/protein kinase C/calcium-dependent nitric oxide synthase signaling pathway, increasing cell proliferation. Carbachol also stimulates the formation of tumor blood vessels and the expression of vascular endothelial growth factor A, concomitantly with tumor cell migration and matrix metalloproteinase-9 expression and activity[148,149]. Similarly to that reported in murine normal mammary cells, the non-tumorigenic human breast cell line MCF-10A does not express these receptors[148].

Triple negative breast tumors are characterized by the absence of a specific therapeutic target and bad prognosis. By studying two different human cell lines derived from this type of tumor, at our lab, we documented that both express mAChRs, but while MDA-MB231 cells express the M1, M2, M4 and M5 receptor subtypes, MDA-MB468 express all receptor subtypes including M3 protein[128]. Similarly to that documentated in our previous reports, short-time activation of these cell lines with carbachol stimulated proliferation in a dose-dependent manner. This effect was prevented by the presence of the muscarinic antagonist atropine, confirming the involvement of mAChRs in this effect[128]. In MDA-MB231 cells, we were able to identify the receptor involved in the mentioned effects by using small interfering RNA silencing assays, which revealed that the one responsible for the proliferative actions in this cell line is the M2 subtype[128].

Based on previous reports by other authors and our results proving that long-term and low-concentration treatment with muscarinic agonists induces a decrease in cell viability, we next decided to focus on mAChRs as therapeutic targets for breast cancer treatment in triple negative tumors[128,129].

To assign more specificity to mAChRs as blanks of action in anti-tumor therapy, it must be taken in mind that non-tumorigenic human mammary cells, like MCF-10A, or breast samples from patients with benign pathology (fibroadenoma) lack expression of mAChRs and, as a consequence, they are not sensitive to the treatment with muscarinic agonists. However, when transfected with mAChRs, normal cells acquire the ability to respond to muscarinic treatment[129]. All these results highlight the relationship between mAChRs and breast cancer development and treatment.

CHEMOTHERAPEUTIC DRUGS INTERACTING WITH MACHRS

Many chemotherapeutic drugs are used in breast cancer treatment, but only two of them are able to bind with the active site of mAChRs: paclitaxel[82] and doxorubicin [150]. Both induce an inhibitory effect on cell proliferation *in vitro* similar to that observed with the cholinergic agonist carbachol[128].

Paclitaxel

Paclitaxel is a drug of first choice in the treatment of breast cancer. It is a taxane derived from the tree *Taxus brevifolia*[151]. This drug was approved by the Food and Drugs Administration (FDA) for the treatment of ovarian cancer in 1992, for advanced stages of breast cancer in 1995[152], and for the early stages of breast cancer in 2001 [153]. Paclitaxel is a diterpenoid pseudoalkaloid made up of an N-benzoyl phenylisoserine group and a taxane ring. In contrast with other taxanes, paclitaxel has a lateral complex chain connected to its taxane ring in C-13, which gives this drug its antitumor activity[154]. Its molecular formula is $C_{47}H_5NO_{14}$ and its chemical structure is described in Figure 2.

Despite the extended use of paclitaxel in anti-tumor treatment, it has been reported that its administration at therapeutic concentrations (10^{-6} mol/L approximately) causes

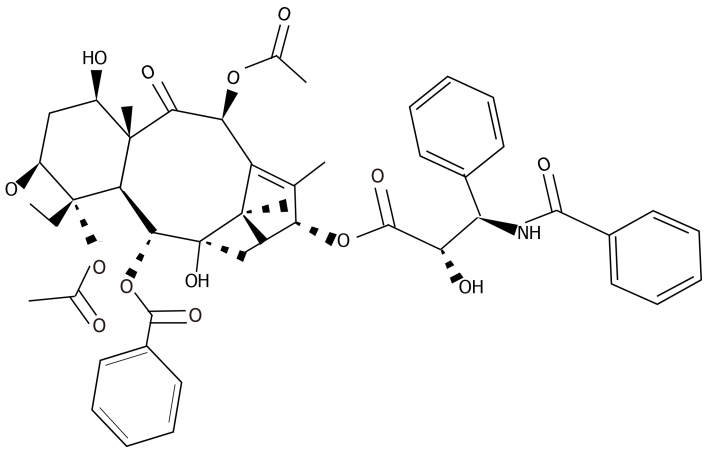


Figure 2 Chemical structure of paclitaxel.

adverse effects like hepatotoxicity, leukopenia, neutropenia, anemia, thrombocytopenia, neurotoxicity, vomits, alopecia, fatigue, mucositis and diarrhea[155-158]. In addition to the low-efficiency synthesis of paclitaxel and its side effects, another problem is its insolubility in water, which affects its bioavailability. Thus, to improve its availability, it is dissolved in polyoxyethylated castor oil as a vehicle (Cremophor EL); however, this oil has been associated with several anaphylactic reactions like dyspnea with bronchospasm, hypotension and urticaria since it activates the complement system[159]. Because of this, new technologies have been developed to improve the biodistribution of this taxane, and alternative ways of administration like paclitaxel binded to albumin[160,161], paclitaxel-loaded PEG-vitamin E nanoparticles [162,163] and micellar paclitaxel[164,165] have been proposed.

The conventional dose of paclitaxel administered to treat breast cancer is 175 mg/m^2 skin by intravenous inoculation for 3 h every 21 d in 6 cycles[166] and its halflife time after inoculation is 4.9 ± 3.6 h[167]. After one administration, most of the paclitaxel in the circulation binds to proteins and its systemic clearance is 700 mL/min on average [168]. Approximately 90% of the drug is degraded by hepatic metabolism by P-450 isoenzymes (CYP3A and CYP2C) and is excreted by feces, while the other 10% is excreted by urine without alterations[168-170].

Paclitaxel exerts its anti-tumor effects through different mechanisms. One of these is by acting as a cytostatic drug that binds to the β subunit of tubulin and stabilizes the polymerized microtubules, inhibiting their depolymerization[171,172]. Consequently, cells lose their ability to divide due to insufficient requirements in the G2/M mitotic control checkpoint[173]. The prolonged arrest of mitosis eventually leads to cell death [174].

Besides its antimitotic effect, paclitaxel also acts in the immune system since its administration is associated with the change in the macrophage phenotype from an M2 to an M1 profile through TLR4 (toll-like receptor 4) activation[175]. Millrud *et al* [176] reported that, in the presence of paclitaxel, primary human monocytes turn into proinflammatory M1 macrophages, contributing to tumor eradication.

Also, at low doses, paclitaxel can induce apoptosis in tumor cells by inactivating the B-cell lymphoma 2 protein (Bcl-2)[177-179]. Additionally, it has been described that this cytostatic drug modulates several non-coding RNAs that have a regulatory function over genes related to tumor progression[180]. Moreover, paclitaxel can modulate the release of the apoptogenic factor cytochrome that can trigger programmed cell death[181,182]. The dysregulation of these and other parameters can lead to resistance to paclitaxel treatment.

One interesting point that we have analyzed in our laboratory is the ability of paclitaxel to interact with mAChRs in a specific manner[126]. We proved that paclitaxel displaces the binding of [3H]-QNB to mAChRs expressed in murine mammary adenocarcinoma cell lines in a manner similar to that of atropine. Moreover, when we treated these cells with paclitaxel at very low concentrations (10^{-11} mol/L), we reported an inhibitory effect on tumor cell proliferation. This effect was prevented by the previous treatment of cells with atropine, confirming the participation of mAChRs in this action[126].

Doxorubicin

The other chemotherapeutic drug frequently used in breast cancer treatment is doxorubicin[183,184], which is an antibiotic that belongs to the family of anthracyclines and is produced by the bacterium *Streptomyces peucetius*. It was approved by the FDA in 1974 for metastatic breast cancer treatment[185]. Its molecular formula is $C_{27}H_{29}NO_{11}$ and its chemical structure is shown in Figure 3.

The treatment with doxorubicin causes many side effects, including cardiotoxicity [186], infertility[187], genotoxicity[188], amenorrhea[189], thrombophlebitis[190] and lung embolism[191]. Regarding cardiotoxicity, the additional challenge is that it can occur 10 years after chemotherapy treatment, appearing as a progressive congestive cardiac failure secondary to a non-ischemic dilated cardiomyopathy, and is irreversible and usually fatal[192,193]. Although many authors indicate that this effect is mediated by cardiac adrenergic interactions[194-196], Chugun *et al*[197] pointed out that it would be due to muscarinic interactions by modulating the ionotropic effect of carbachol.

In breast cancer, doxorubicin is administered in 60/75 mg/m^2 per dose intravenously in one inoculation every 21 d. Its half life time is approximately 48 h[198] and its systemic clearance is between 700 and 1250 mL/min[199,200]. Most of the doxorubicin is degraded by hepatic metabolism and the unaltered drug and its metabolite are excreted mainly by the gallbladder and in a small fraction by the kidneys[201].

Doxorubicin exerts its chemotherapeutic effect by three mechanisms. The first one is the inhibition of the enzyme topoisomerase II-α, which regulates the superhelical state of DNA and has a structural function regulating the tension of the DNA strands[202]. Doxorubicin stabilizes the binding of topoisomerase II-α to the cleaved DNA, preventing the replication process and leading the cell to apoptosis[203]. Given that tumor cells present a higher proliferative rate, the expression of topoisomerase II-α is high, producing a greater selectivity of doxorubicin for these cells[204].

The second mechanism of action also affects directly the DNA strands. Doxorubicin intercalates into DNA, inhibiting the activity of topoisomerase II-α, which in turn causes the inhibition of DNA synthesis[205]. Moreover, doxorubicin intercalation causes the break of the DNA in double-strand fragments and chromatin condensation, which leads to an increase in apoptosis[206]. This antibiotic can also intercalate in RNA, inhibiting the activity of RNA polymerase, although it has more affinity for DNA[207].

Finally, the third mechanism of action of doxorubicin is the production of free radicals. Doxorubicin can act as an electron acceptor, transforming its quinone to a semiquinone-free radical, which causes oxidative damage and induces the cleavage and degradation of DNA. This is a mitochondrial reaction catalyzed by the enzyme cytochrome P450 reductase in the presence of NADH dehydrogenase[208]. It has also been described that the free molecular iron can interact with doxorubicin, forming toxic free radicals and reactive nitrogen species that increase nitrosative stress and mitochondrial dysfunction, promoting apoptosis[209].

Interestingly, doxorubicin can also interact with mAChRs. It has been demonstrated that doxorubicin displaces the [3H]-QNB binding in the left atrial muscle of guinea pig hearts in a concentration-dependent manner, similarly to atropine, indicating that doxorubicin can also bind to mAChRs[150,197]. Additionally, in triple negative human tumor cells, we determined that doxorubicin exerts an inhibitory effect on proliferation, comparable to that induced by carbachol[128].

Multidrug resistance

One of the most important causes in the failure of chemotherapy not only in breast cancer treatment but also in other cancers is the appearance of resistance. It can appear as a primary form, when it is present before the treatment, or acquired, when it develops after the exposure to a drug[210,211].

To improve the efficacy of chemotherapeutic agents in the treatment of breast cancer, it is important to define the mechanisms underlying resistance in this type of tumor. In breast cancer, resistance is multifactorial and implies different mechanisms and genes that exert their effects either together or separately, leading to a reduction or an inhibition of the effect of different drugs.

Regarding the factors that modulate the resistance to paclitaxel or doxorubicin, some of them are common to both drugs and others are specific to each of them. The main factors in common are the expression of drug extrusion pumps, noncoding RNAs, Bcl-2 and p53. Drug extrusion pumps are also known as ATP binding cassette (ABC) transporters. They are located in the cytoplasmic membrane and modulate the

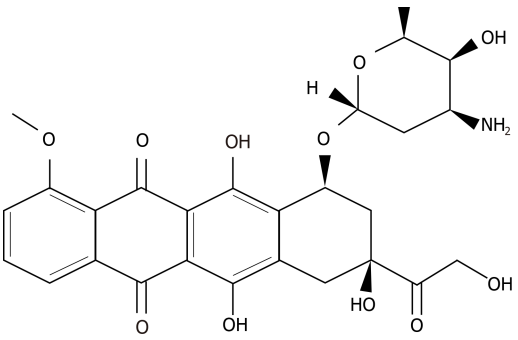


Figure 3 Chemical structure of doxorubicin.

time that the drug is present inside the cell by controlling its active transport. The expression and activity of these pumps can be modified by different factors, including the chemotherapy treatment. Amawi *et al*[212] reported that paclitaxel treatment exerts an increase in pump activity and expression in different breast cancer cell lines, whereas Cox and Weinman[213] reported similar results in hepatocellular carcinomas treated with doxorubicin. In both cases, the increase in the expression and activity of these pumps reduced the effectiveness of the treatment. Regarding the other factors that modulate the action of paclitaxel or doxorubicin, noncoding RNAs, also called miRNA, can modulate the expression of genes that regulate apoptosis and cell survival, increasing the sensitivity or the resistance to the chemotherapeutic treatment [214-216], whereas Bcl-2 and p53 expression proteins regulate tumor growth and are important tumor markers that can be modulated by chemotherapeutic drugs, increasing the resistance to treatment[217,218].

Among paclitaxel resistance factors, the most relevant are changes in the cytoskeletal dynamics and kinetic degradation of the taxane structure. Several tumor cells express high levels of βIII-tubulin that are related to resistance to paclitaxel treatment[219,220]. When this expression is down-regulated, cells recover sensitivity to paclitaxel[221]. In this regard, Wang *et al*[222] described an increase in the expression of βIII-tubulin, which induces resistance because higher concentrations of the drug are needed to stabilize microtubules and block cell division.

Regarding the proteins that modulate cytoskeletal dynamics, many authors have established that an increase in the resistance to paclitaxel treatment is related to an over-expression of stathmin[223], septin[224], tubulin binding cofactor C[225] and BRCA1[226] proteins.

It is also known that enzymes of the cytochrome P450 subfamily 3A and 2C play a major role in the metabolism of taxane anticancer agents. The expression of these enzymes in solid tumors may thus play a role in the *in situ* metabolism of drugs as well, potentially affecting the intrinsic susceptibility of these tumors to taxane. An abnormal up-regulation of enzyme activity or expression reduces the halflife time of paclitaxel and as a consequence its efficacy in cancer patients[227].

Regarding doxorubicin, the expression of topoisomerase II-α and FOXO3 is mentioned as a main aspect in the mechanism of resistance to this drug. Doxorubicin exerts part of its chemotherapeutic effect by the binding to topoisomerase II-α, which produces DNA structure stabilization and induces apoptosis. Wang *et al*[222] reported that a down-regulation in topoisomerase II-α expression induces resistance to doxorubicin treatment in human breast tumor cells MCF-7. In human malignant breast samples, O'Malley *et al*[228] demonstrated that topoisomerase II-α expression is a good marker to determine the resistance to doxorubicin since cells with higher levels of this enzyme are more sensitive to this anthracycline.

FOXO3 is a factor associated with longevity due to its antioxidant effect[229]. In this regard, Gomes *et al*[230] reported that the administration of doxorubicin in breast cancer patients can increase FOXO3 expression, inducing resistance to treatment and making it a marker of bad prognosis.

Other side effects

Besides the previous points, it must be taken into account that the treatment with these drugs can stimulate the development of metastases. Regarding this matter, Karagiannis *et al*[231] demonstrated that the *in vivo* administration of doxorubicin or paclitaxel to murine breast tumor bearers promotes the formation of micrometastases in the lung, which is the first step necessary to produce tumor cell intravasation and

the subsequent formation of metastases. Moreover, Daenen *et al*[232] established that paclitaxel treatment also increases vascular endothelial growth factor receptor-1 expression in lung endothelial cells, which stimulates the adhesion of circulating tumor cells and the subsequent formation of metastases.

Additionally, conventional therapeutic treatment with doxorubicin or paclitaxel can affect normal cells, inducing cell death. We and other authors have demonstrated that, at therapeutic concentrations, paclitaxel besides reducing cell viability in human breast tumor cells MCF-7 and MDA-MB231 also causes cell death in normal mammary cells MCF-10A[128,233-235].

METRONOMIC THERAPY

The conventional treatment usually applied to cancer patients considers the administration of chemotherapeutic drugs at the maximum tolerated dose. Because of all the undesirable effects caused, patients need a long interval of time between cycles of treatment so that normal tissues can recover. The usage of the maximum tolerated dose is useful in tumors that rarely have a complex network of activating mutations, like acute lymphoblastic leukemia or testicular cancer. However, in other tumors like breast tumors, this conventional treatment is less effective mainly because these tumors permanently modulate the tumor microenvironment. In addition, as mentioned above, besides the toxicity induced by the administration of the maximum tolerated dose in breast cancer, development of resistance to treatment is frequently described[236,237].

One of the strategies to reduce side effects and the resistance to anti-tumor drugs is the administration of minimal doses of one or more chemotherapeutic agents, which, administered in a continuous regime or with short time intervals, improves the results of treatment. This strategy is known as metronomic therapy and its main aim is to reduce the toxicity and to increase the quality of the patients' lives.

Metronomic therapy may be effective to inhibit tumor progression through different mechanisms that modulate not only tumor cell death but also the cancer stem cell population involved in tumor generation and metastasis[129,236]. Folkins *et al*[238] demonstrated that metronomic therapy with cyclophosphamide could reduce the growth of human glioma spheroids, a cancer stem cell marker. Vives *et al*[239] reported that the same treatment reduces the number of cancer stem precursor cells in human pancreatic tumor. Similarly, in our laboratory, we demonstrated that the treatment of human breast cancer cells MCF-7 with paclitaxel plus carbachol in a metronomic schedule causes a decrease in the cancer stem cell population[129].

The target of this kind of therapy is not only tumor cells but other cells of the tumor microenvironment[236,240-242]. Several authors have demonstrated that some chemotherapeutic drugs administered at low doses can inhibit the synthesis of pro-angiogenic factors produced by endothelial cells, which are necessary for tumor growth[236].

In addition, metronomic therapy is effective to modulate the activity of the immune system, by decreasing the number of regulatory T cells and increasing the population of cytotoxic T lymphocytes and natural killer cells[243,244].

Metronomic therapy is usually linked to repurposing drugs. The latter refers to the assignation of new uses for drugs usually administered to treat diseases other than cancer[245]. In oncology, there is an increasing interest in the prescription of non-cancer drugs for cancer treatments due to the knowledge of their pharmacokinetics/dynamics and side effects, and because most of them are available at low cost[246].

Considering previous results obtained in our laboratory, we have recently proposed the administration of low doses of paclitaxel or doxorubicin combined with low doses of carbachol, a non-selective muscarinic agonist, or arecaidine, an M2 selective agonist, in a metronomic schedule to effectively reduce breast tumor cell viability[128]. As shown in Table 2, the effect of metronomic combinations is similar to that obtained with paclitaxel or doxorubicin administered at therapeutic concentrations (10^{-6} mol/L). These results position muscarinic agonists in the spectrum of repurposing drugs.

Our results focused on the presence of mAChRs in tumor cells and their absence in normal cells, giving specificity to this type of anti-tumor therapy, and indicating that it also prevents cytotoxic actions in normal cells, which could be an indicator of reduced adverse effects (Table 2).

It is important to mention that the treatment with paclitaxel plus carbachol not only reduces tumor cell viability but also prevents other important steps of tumor progression. This therapy diminishes tumor cell migration, cancer stem cell

Table 2 Effect of metronomic chemotherapy targeting muscarinic acetylcholine receptors on breast tumors

Murine breast malignant cells	Murine breast not malignant cells
Paclitaxel (10-11 mol/L) + Carbachol (10-9 mol/L)[126]	
LM2: 68.2 ± 5.1[b]	NMuMG: 6.8 ± 6.6
LM3: 64.6 ± 6.5[b]	
Human breast malignant cells	**Human breast not malignant cells**
Paclitaxel (10-9 mol/L) + Carbachol (10-11 mol/L)[127]	
MCF-7: 45.6 ± 5.8[a]	MCF-10A: 1.9 ± 0.3
Paclitaxel (10-9 mol/L) + Carbachol (10-10 mol/L)[128]	
MDA-MB468: 33.4 ± 2.5[c]	MCF-10A: 2.9 ± 3.1
Paclitaxel (10-9 mol/L) + Arecaidine propargyl ester (10-7 mol/L)[128]	
MDA-MB468: 26.9 ± 3.6[c]	MCF-10A: 3.3 ± 6.2
Paclitaxel (10-8 mol/L) + Carbachol (10-11 mol/L)[128]	
MDA-MB231: 36.8 ± 6.2[c]	MCF-10A: 3.1 ± 6.2
Doxorubicin (10-8 mol/L) + Carbachol (10-11 mol/L)[128]	
MDA-MB231: 35.3 ± 0.8[c]	MCF-10A: 6.1 ± 3.1
Paclitaxel (10-8 mol/L) + Arecaidine propargyl ester (10-5 mol/L)[128]	
MDA-MB231: 35.8 ± 3.1[c]	MCF-10A: 8.2 ± 6.5
Doxorubicin (10-8 mol/L) + Arecaidine propargyl ester (10-5 mol/L)[128]	
MDA-MB231: 33.3 ± 2.1[c]	MCF-10A: 7.8 ± 6.8
Paclitaxel (10-6 mol/L)[128]	
MDA-MB231: 38.0 ± 6.1[c]	MCF-10A: 33.3 ± 1.5[c]
Doxorubicin (10-6 mol/L)[128]	
MDA-MB231: 41.1 ± 2.3[c]	MCF-10A: 45.7 ± 1.8[c]

[a]$P < 0.01$.
[b]$P < 0.001$.
[c]$P < 0.0001$ *vs* control (untreated cells).

percentage, neoangiogenesis and the expression of the drug extrusion pump ABCG2 [128].

CONCLUSION

The research about new antitumor therapies with drugs that increase beneficial actions and reduce adverse effects is a challenge to improve breast cancer patients' lives. The usage of repurposing drugs, like the muscarinic agonist carbachol, which synergizes the action of traditional anti-tumor drugs might be an alternative schedule focused on mAChRs as new therapeutic targets. The presence of these receptors at high concentrations not only in breast tumors but also in other types of tumor could help to find a more specific and less aggressive manner to treat cancer patients. On the other hand, metronomic therapy is effective to kill tumor cells without affecting normal cells and also decreases pathological neo-angiogenesis and the expression of drug extrusion proteins. The latter could prevent the appearance of resistance reported in conventional chemotherapy. More *in vivo* experiments are needed to confirm the effectiveness of this treatment in breast cancer models and to gain information to discard systemic adverse reactions.

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MINIREVIEWS

Review of 10 years of research on breast cancer patients: Focus on indoleamine 2,3-dioxygenase

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Abstract

Therapeutic manipulation of the immune system in cancer has been an extensive area of research in the field of oncoimmunology. Immunosuppression regulates antitumour immune responses. An immunosuppressive enzyme, indoleamine 2,3-dioxygenase (IDO) mediates tumour immune escape in various malignancies including breast cancer. IDO upregulation in breast cancer cells may lead to the recruitment of regulatory T (T-regs) cells into the tumour microenvironment, thus inhibiting local immune responses and promoting metastasis. Immunosuppression induced by myeloid derived suppressor cells activated in an IDO-dependent manner may enhance the possibility of immune evasion in breast cancer. IDO overexpression has independent prognostic significance in a subtype of breast cancer of emerging interest, basal-like breast carcinoma. IDO inhibitors as adjuvant therapeutic agents may have clinical implications in breast cancer. This review proposes future prospects of IDO not only as a therapeutic target but also as a valuable prognostic marker for breast cancer.

Key Words: Indoleamine 2,3-dioxygenase; Breast cancer; Therapeutic target; Prognostic marker; Immune responses; Immune escape

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INTRODUCTION

Breast cancer is the most common cancer in women worldwide. A variety of genetic and non-genetic factors can be linked to breast cancer. Recent emerging epidemiologic, preclinical, and clinical data suggest the key role of the immune system in the aetiology of breast cancer[1]. The current understanding of the molecular and cellular mechanisms underlying cancer development suggests that immune cells functionally regulate epithelial cancer development and progression[2]. The innate and adaptive immune systems play a role in preventing relapse in breast cancer[1]. Lymphocytes, including T cells, T-regs, natural killer (NK) cells, and their cytokine release patterns are associated with both primary prevention and relapse of breast cancer[1,3]. Hence, breast cancer prognosis may be related to the functional status of the immune system.

Breast cancer cells can evade immune responses, by various immunosuppressive mechanisms, such as the upregulation of indoleamine 2,3-dioxygenase (IDO/IDO-1)[4,5]. IDO is a heme-containing immunosuppressive enzyme, that degrades the essential amino acid L-tryptophan into kynurenine[6]. IDO is involved in the immune homeostasis and immune-related functions not only during pregnancy but also in chronic inflammatory diseases and tumour immune-escaping mechanisms[7-9]. IDO is chronically triggered in cancer patients[10]. Deprivation of tryptophan directly affects the cytotoxicity of T cells. In addition, the toxic metabolites produced from tryptophan degradation directly induce T cell apoptosis *in vitro*[9]. IDO may inhibit T cell immunity by inducing the differentiation and maturation of T-regs[11]. IDO overexpression induces immunosuppression and tolerance[8]. IDO-expressing cells are found at several sites of immune tolerance, including thymus, placenta, anterior chamber of the eye, mucosa of the gut and epididymis[12-14]. Human monocyte-derived macrophages and dendritic cells (DCs) express IDO[15,16]. IDO-expressing DCs are found in breast tumour tissue as well as draining lymph nodes of patients with breast cancers[17]. IDO expression may suppress immune responses by blocking NK cells (Figure 1)[18]. However, the immunosuppressive role of IDO in tumour immunology and its associations with other tolerogenic mechanisms have only recently begun to be elucidated. The molecular mechanisms underlying tumour immune escape are currently the topic of active research. In this review, we examine the potential role of IDO as a prognostic marker and therapeutic target in breast cancer patients.

IMPACT OF IDO ON BREAST CANCER PATIENTS

It has been established that IDO in the tumour microenvironment has the capacity to inhibit antitumour immunity and promote metastasis, both hallmarks of cancer[19]. It is evident that IDO is consistently and robustly expressed in breast cancer[20]. IDO is suggested to play a pivotal role in the pathogenesis of breast cancer (Table 1). In 2011, Yu *et al*[21] observed that the upregulation of IDO in primary breast cancer might inhibit the local immune response by the infiltration of T-regs into the tumour microenvironment thereby promoting metastasis[21]. In 2013, Yu *et al*[22] further investigated IDO expression in myeloid-derived suppressive cells in breast cancer and observed that STAT3-dependent IDO expression induced the immunosuppressive effects of myeloid derived suppressor cells in breast cancer[22]. IDO has recently received more attention because of its involvement in regulating angiogenesis[23]. Wei *et al*[23] studied the effects of IDO on microvessel density and reported that high IDO expression is associated with microvessel density; causes a poor prognosis and subsequently promotes angiogenesis in breast cancer[23].

Table 1 Review of ten years of research on indoleamine 2,3-dioxygenase in breast cancer patients

Year	Ref.	Population	Description
2011	Yu *et al*[21]	Chinese (*n* = 26)	IDO upregulation inhibits local immune responses by infiltration of T-regs in the tumour microenvironment and promotes metastasis in breast cancer
2011	Lyon *et al*[27]	American (*n* = 33)	Increased tryptophan degradation may occur in women with early-stage breast cancer
2012	Jacquemier *et al*[31]	French (*n* = 1749)	Immunodetection of IDO-positive cells may be used for diagnosis of medullary breast cancer. IDO has a prognostic significance in basal like breast cancer
2013	Soliman *et al*[24]	American (*n* = 203)	IDO expression was higher in ER^+ breast cancer than ER^- breast cancer
2013	Yu *et al*[22]	Chinese (*n* = 85)	STAT3-dependent IDO expression induces immunosuppressive effects of MDSCs in breast cancer
2014	Bi *et al*[37]	Chinese (*n* = 110)	IDO and EGFR may serve as a potential biomarkers for breast cancer prognosis and treatment
2014	Isla Larrain *et al*[32]	Argentinian(*n* = 91)	IDO was expressed in a TNBC subgroup and was involved in the tumour immune escape
2015	Salvadori *et al*[33]	Brazilian (*n* = 20)	IDO inhibitor when combined with paclitaxel may be used as a new therapeutic strategy for breast cancer
2017	Kim *et al*[30]	South Korean (*n* = 200)	IDO might be an effective immunotherapeutic target in TNBC
2017	Dewi *et al*[25]	German (*n* = 15)	IDO-1 expression in ER^- breast cancer may be associated with poor prognosis. IDO-1 maybe a promising therapeutic target for ER^- breast cancer
2017	Carvajal-Hausdorf *et al*[36]	American (*n* = 362)	IDO-1 quantification has potential to differentiate a population that might get an advantage from IDO-1 blockade
2017	Li *et al*[38]	Chinese (*n* = 46)	Expression of IDO and IL-6 is associated with advanced breast cancer and poor response to neoadjuvant chemotherapy
2018	Ye *et al*[34]	Chinese (*n* = 963)	IDO and programmed cell death protein-1 pathways might be an effective therapeutic approach in breast cancer treatment
2018	Wei *et al*[23]	Chinese (*n* = 65)	IDO may induce angiogenesis in breast cancer, providing a molecular or gene therapy target for angiogenesis inhibition
2018	Li *et al*[39]	Chinese (*n* = 44)	Tumour-infiltrating T-regs, MDSCs and IDO expression may be used as a prognostic marker for the outcome of neoadjuvant chemotherapy
2018	Dill *et al*[29]	American (*n* = 281)	IDO expression in high-grade,TNBC is associated with PD-L1 co-expression
2019	Asghar *et al*[28]	Pakistani (*n* = 100)	IDO expression in TNBC may suggest its role in disease pathogenesis
2019	Onseti *et al*[26]	Belgian (*n* = 202)	Kynurenine/tryptophan ratio in plasma might differentiate breast cancer patients from healthy controls
2019	Asghar *et al*[35]	Pakistani (*n* = 100)	IDO expression is associated with COX-2 expression in breast cancer patients
2019	Zhao *et al*[40]	Chinese (*n* = 53)	IDO expression and activity is linked with advanced breast cancer and poor response to neoadjuvant chemotherapy
2020	Wei *et al*[41]	Chinese (*n* = 77)	IDO and tumour infiltrating immune cells can help to evaluate the prognosis of breast cancer patient

IDO: Indoleamine 2,3-dioxygenase; T-regs: regulatory T cells; ER: Estrogen receptor; TNBC: triple-negative breast cancer; STAT3: Signal transducer and activator of transcription 3; MDSCs: Myeloid-derived suppressor cells; EGFR: Epidermal growth factor receptor; COX-2: Cyclooxygenase-2.

Soliman *et al*[24] also examined IDO expression in breast cancer patients (*n* = 203). IDO overexpression was detected in ER^+ tumours but not ER^- tumours. Overall survival was better in ER^+ patients with high IDO expression. This study provided a new prospective for ongoing clinical trials of IDO inhibitors in metastatic breast cancer. They proposed further studies to understand the complex role of IDO in the natural progression of breast cancer at different stages of the disease[24]. Another study published by Dewi *et al*[25] demonstrated that increased IDO expression in ER^- breast cancer might influence its malignant phenotype and result in a poor prognosis [25].

The kynurenine to tryptophan (Kyn/Trp) ratio is used to measure IDO enzymatic activity. Onesti *et al*[26] measured IDO activity in breast cancer patients (*n* = 202) with all subtypes and healthy controls (*n* = 146). They reported that the Kyn/Trp ratio might differentiate breast cancer patients from healthy controls[26]. A similar study conducted by Lyon *et al*[27] compared the levels of tryptophan degradation in women

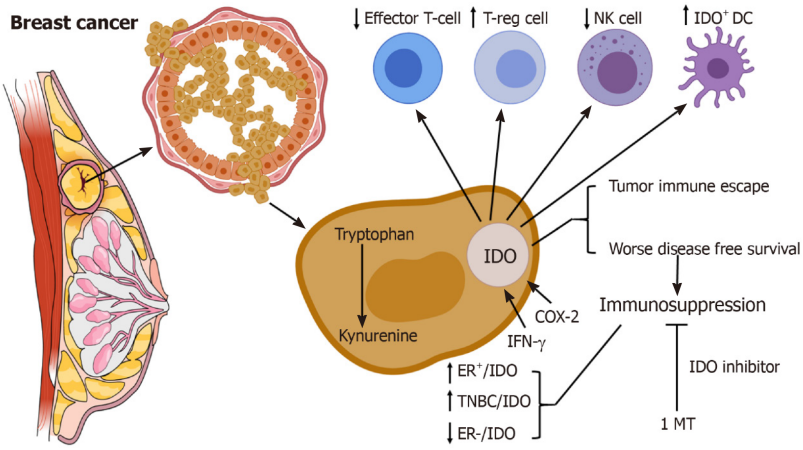


Figure 1 Indoleamine 2,3-dioxygenase inhibition as potential immunotherapeutic strategy for breast cancer. Indoleamine 2,3-dioxygenase (IDO) is heme-containing immunosuppressive enzyme. IFN-γ is a potent inducer of IDO. Cyclooxygenase-2 expression by tumour cells stimulates intrinsic tumour expression of IDO. IDO degrades tryptophan into kynurenines. Deprivation of tryptophan directly affects the cytotoxicity of T cells. IDO may inhibit T cell immunity by inducing the differentiation and maturation of T-regs. IDO expression could suppress immune responses by blocking natural killer cells. IDO$^+$ dendritic cells are found in breast tumour as well as axillary lymph nodes of these patients. High IDO expression is observed in ER$^+$ tumours than ER$^-$ tumours. IDO is expressed in a triple-negative subgroup. IDO expression is associated with tumour immune escape and overall survival of the patients. 1-MT is the pharmacological inhibitor of IDO. IDO: Indoleamine 2,3-dioxygenase; COX-2: Cyclooxygenase-2; NK: Natural killer; ER: Estrogen receptor.

with or without breast cancer and observed an increased Kyn/Trp ratio in women with breast cancer. Keeping in view the multifactorial role of IDO, the authors suggested that further research was necessary to determine the relationships among these important biological factors and neuropsychiatric symptoms in women with breast cancer[27].

IDO IN TRIPLE-NEGATIVE BREAST CANCER

Due to advancements in early diagnosis and treatment of breast cancer, the overall survival of patients has significantly improved over the years. Nevertheless, triple-negative breast cancer (TNBC) is a more aggressive tumour than other breast cancers [28]. Our recent data showed high IDO expression in TNBC patients. Furthermore, we observed that high IDO expression was significantly correlated with decreased overall survival[28]. Dill *et al*[29] also observed IDO expression among high-grade TNBC. In addition, they determined that IDO expression was associated with PD-L1 co-expression. They suggested clinical trials to assess the effectiveness of IDO inhibition relative to IDO expression as well as its role when combined with anti-programmed cell death protein 1 (PD-1)/PD-L1 immunotherapy[29]. Another study by Kim *et al*[30] was conducted to evaluate the clinical and pathological characteristics of an IDO-expressing TNBC subset, and the authors observed that IDO positivity was associated with the basal-like phenotype. They also suggested the role of IDO blockade in the treatment of TNBC patients[30].

Among the molecular subtypes of breast cancer, basal-like breast carcinoma (BLBC) has the poorest outcomes[31]. Jacquemier *et al*[31] determined that IDO was overexpressed at the transcriptional and translational levels in a subset of TNBC. They elaborated that IDO overexpression was correlated with morphological medullary features and had autonomous prognostic significance in BLBC. Medullary breast carcinoma (MBC) had a better prognosis than non-MBC, but IDO was overexpressed at the mRNA level in BLBC and MBC compared with non-MBC. IDO expression is thus correlated with tumour infiltrating lymphocytes, which are present in both MBC and BLBC. IDO overexpression in BLBC with a favourable prognosis may be due to kynurenines, which have the capacity to induce apoptosis in lymphocytes as well as tumour cells[31]. Another study revealed that IDO was mostly expressed in the TNBC subtype. The same authors also observed IDO expression in breast cancer and circulating microvesicles from breast cancer patients with advanced stages[32].

THERAPEUTIC AND PROGNOSTIC SIGNIFICANCE OF IDO

IDO inhibitors have therapeutic significance when given in combination with chemotherapeutic agents for breast cancer treatment[33]. An IDO inhibitor (1-methyl-DL-tryptophan) in combination with paclitaxel may be a new therapeutic strategy for breast cancer[33]. Several studies support this hypothesis; for instance, Ye *et al*[34] investigated the association between IDO and PD-1 in the tumour microenvironment and in tumour-draining lymph nodes in breast cancer patients. They observed a positive association between the expression of IDO and PD-1. The team also proposed that inhibiting both of these pathways might act as a novel therapeutic strategy in breast cancer treatment[34]. Another study published by Asghar *et al*[35] reported that high IDO expression was correlated with high cyclooxygenase-2 expression in breast cancer patients. It was suggested that the simultaneous targeting of cyclooxygenase-2 and IDO may have potential for treatment of breast cancer[35]. Carvajal-Hausdorf *et al*[36] observed that the IDO protein was expressed in hormone receptor-positive breast cancer. Furthermore, IDO was negatively correlated with B-cell infiltration in tumours, and high IDO expression was associated with poor overall survival. The authors proposed that IDO quantification has the potential to differentiate a population that might obtain an advantage from IDO-1 blockade[36].

IDO has not only therapeutic significance but also prognostic significance. Bi *et al*[37] observed the co-expression of IDO and epidermal growth factor receptor (EGFR) in breast cancer and suggested that IDO and EGFR may serve as potential biomarkers for breast cancer prognosis and treatment[37]. Another study conducted by Li *et al*[38] in 2017, aimed to investigate the co-expression of IDO and interleukin-6 in breast cancer patients prior to neoadjuvant chemotherapy. They observed that IDO and interleukin-6 expression was related to advanced breast cancer and a poor response to neoadjuvant therapy[38]. In 2018, Li *et al*[39] further explored whether tumour-infiltrating T-regs, myeloid-derived suppressor cells and IDO expression may be used as prognostic markers for the outcome of neoadjuvant chemotherapy[39]. Additionally, Zhao *et al*[40] observed that high IDO expression and activity were associated with advanced disease, a poor prognosis and chemoresistance in breast cancer[40]. In 2020, Wei *et al*[41] identified tumour infiltrating immune cells, and IDO and PDL-1 expression in breast cancer patients. The authors proposed that IDO in combination with tumour infiltrating immune cells might help to assess the prognosis of patients with breast cancer[41].

FUTURE PROSPECTS

IDO is involved in the regulation of the immune system. Upregulated IDO is associated with a poor prognosis in various cancers[42-44], but in the case of BLBC, high IDO expression indicates a favourable prognosis[31]. Another study revealed improved overall survival among ER^+ breast cancer patients with high IDO expression [24]. However, several studies carried out regarding IDO involvement in breast cancer showed that IDO overexpression was associated with breast tumour growth and metastasis[4,21,45,46]. In view of the contradictory findings, further studies are therefore required to understand the complex role of IDO in breast cancer. Identification of more efficient and less toxic IDO inhibitors is urgent. Currently, two IDO inhibitors are in a clinical development stage as immunotherapeutic agents in breast cancer treatment. These inhibitors are Indoximod (NLG2101) developed by NewLink Genetics[47] and INCB024360 developed by Incyte[48,49]. Two therapies have been exclusively designed to treat HER2-positive breast cancer in combination with the AD.p53 DC vaccine and docetaxel (NCT01042535 and NCT01792050 respectively)[50]. IDO inhibitors as adjuvant therapeutic agents may have clinical implications in breast cancer[33]. Targeted IDO inhibition using nanoparticles may provide a better outcome. Tryptophan-2,3-dioxygenase (TDO) has biochemical activity similar to that of IDO[51,52]. Apart from IDO, another isoform IDO-2 has also been discovered[53,54]. Both IDO-2 and TDO are involved in the degradation of tryptophan[55]. Future studies should focus on the role of IDO-2 and TDO in breast cancer.

CONCLUSION

The therapeutic implications of IDO are unquestionable but its potential as a prognostic biomarker may have significant outcomes.

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MINIREVIEWS

Imaging diagnosis of bronchogenic carcinoma (the forgotten disease) during times of COVID-19 pandemic: Current and future perspectives

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Abstract

Patients with bronchogenic carcinoma comprise a high-risk group for coronavirus disease 2019 (COVID-19), pneumonia and related complications. Symptoms of COVID-19 related pulmonary syndrome may be similar to deteriorating symptoms encountered during bronchogenic carcinoma progression. These resemblances add further complexity for imaging assessment of bronchogenic carcinoma. Similarities between clinical and imaging findings can pose a major challenge to clinicians in distinguishing COVID-19 super-infection from evolving bronchogenic carcinoma, as the above-mentioned entities require very different therapeutic approaches. However, the goal of bronchogenic carcinoma management during the pandemic is to minimize the risk of exposing patients to COVID-19, whilst still managing all life-threatening events related to bronchogenic carcinoma. The current pandemic has forced all healthcare stakeholders to prioritize per value resources and reorganize therapeutic strategies for timely management of patients with COVID-19 related pulmonary syndrome. Processing of radiographic and computed tomography images by means of artificial intelligence techniques can facilitate triage of patients. Modified and newer therapeutic strategies for patients with bronchogenic carcinoma have been adopted by oncologists around the world for providing uncompromised care within the accepted standards and new guidelines.

Key Words: COVID-19; Bronchogenic carcinoma; Immune checkpoint inhibitor-related pneumonitis; Prioritizing imaging; Surveillance of lung nodules; Artificial intelligence

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Core Tip: Unprecedented times of a pandemic pose a major challenge in maintaining adequate balance between the risk of contracting deadly coronavirus disease 2019 (COVID-19) against the dire consequences of delaying treatment for a life-threatening malignancy. Cancer survivors and patients represent a vulnerable population for COVID-19 related pulmonary syndrome, which can further complicate respiratory and cardiovascular comorbidities they possess. Risk stratification of bronchogenic carcinoma patients and priority imaging may be applied for optimal use of resources during these uncertain times of COVID-19. Utilizing artificial intelligence and deep learning modules based on pattern recognition of image findings during this pandemic has made a lasting impact across future realms.

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INTRODUCTION

In late December 2019, a lower respiratory tract infection was reported in a cluster of patients from Wuhan City, Hubei Province, China. A novel strain of coronavirus was isolated from the bronchoalveolar lavage of infected patients[1]. On January 9, 2020, the World Health Organization named the febrile illness related pulmonary syndrome as coronavirus disease 2019 (COVID-19)[2]. At the time of writing this article on January 19, 2021, the number of confirmed cases stand at 99737233 with 2137990 reported deaths, according to an online virus tracker created by The Lancet and hosted by Johns Hopkins University[3]. Sudden outbreaks of COVID-19 with critically ill patients have put enormous strain on the limited healthcare resources of many countries and caused increased mortality from the pandemic. As of January 2021, the number of confirmed COVID-19 cases globally is over 95 million, vastly affecting every territory other than a few isolated South Atlantic and Pacific Island states and Antarctica[3].

COVID-19 causes mild infection in the majority (80%) of patients showing gradual recovery in about 2 wk[4]. However, COVID-19 has a predilection for middle aged and elderly populations with highest case fatality (8%-15%) seen in the elderly, aged above 80 years. R_0 (basic reproduction number) that is each infected individual — on average — transmitting new cases of novel coronavirus was estimated to be 2.2 based on a study that examined the first 425 infected cases in Wuhan[5]. The study also calculated the average incubation period in the group to be 5.2 d[5].

CORONAVIRUS AND COVID-19 PNEUMONIA

Six subtypes of coronavirus have infected humans in the past, of which four subtypes have caused mild respiratory symptoms, similar to rhinovirus. The other 2 subtypes which are MERS (Middle East respiratory syndrome)-coronavirus and SARS (severe acute respiratory syndrome)-coronavirus have caused severe forms of infection[6]. Relevant epidemiological history and contact history of within 14 d before disease onset have been of tremendous help in contact tracing and cluster tracing of COVID-19 patients[7]. Based on recent epidemiological studies, the incubation period of COVID-19 was calculated to be 1-14 d[8]. Only a minority of COVID-19 patients develop critical illness leading to cardiopulmonary compromise and eventual mortality. The majority of patients have a good prognosis and recovery. COVID-19 related mortality is commonly noted in elderly population with underlying comorbidities.

SYMPTOMATOLOGY AND COMPLICATIONS OF COVID-19

COVID-19 is a febrile illness associated with dry cough, anosmia and ageusia. Severe cases present with symptoms such as dyspnoea, tachypnoea and severe hypoxemia which can progress to acute respiratory distress syndrome (ARDS) and cardiopulmonary failure. Release of interleukin (IL)-1, IL-6 and tumour necrosis factor-α which are proinflammatory cytokines leads to immune-related pneumonitis of COVID-19 which can progress to profound hypotension, septic shock and acute renal failure[9]. Complications in severe COVID-19 include cardiac failure, metabolic acidosis, rhabdomyolysis, coagulopathy, deep vein thrombosis and pulmonary embolism[10].

EPIDEMIOLOGICAL CHARACTERISTICS OF BRONCHOGENIC CARCINOMA PATIENTS WITH COMORBID COVID-19

Analysis of epidemiological characteristics of 72314 COVID-19 patients in mainland China at "The Chinese Center for Disease Control and Prevention" revealed 107 (0.5%) patients to be affected by cancer[11] and similarly, findings from a study published by Chinese researchers on 1590 COVID-19 patients described 18 (1%) patients with a history of cancer, of which five were diagnosed with bronchogenic carcinoma (5/18, 28%)[12]. Their study also demonstrated a higher risk of accelerated events and deteriorating symptoms more rapidly amongst cancer patients (39%) compared to non-cancerous patients (8%)[12]. Findings from a multicentred study done by Dai *et al*[13] on confirmed COVID-19 patients which included 105 cancer patients and 536 age-matched non-cancerous patients, revealed that patients with cancer had higher risks in all severe outcomes. Haematological cancer, bronchogenic carcinoma or metastatic cancer (stage IV) had the highest frequency of severe outcomes. Based on the findings of above mentioned two cohorts, it would be fair to infer that patients with carcinoma are a susceptible population for contracting COVID-19 related pulmonary syndrome with a higher risk of related complications and a higher mortality rate. When patients with any form of cancer develop COVID-19 super-infection, the findings are of higher grade/severity with rapid deterioration to end stage events. Further complications include the inability of healthcare stakeholders to adequately streamline ample resources for cancer patients and shortage of services for providing timely antineoplastic treatment which results in poor prognosis.

CURRENT PERSPECTIVES

Unprecedented times of a pandemic pose a major challenge in maintaining adequate balance between the risk of contracting deadly COVID-19 against the dire consequences of delaying treatment for a life-threatening malignancy. Radiographic changes may precede symptoms and must be interpreted cautiously during a pandemic like this[14]. Ongoing clinical trials for anti-cancer drugs have drastically slowed down during this pandemic with new clinical trial enrolment temporarily halted. However, if adequate resources are available, clinical trial enrolment should continue in compliance with appropriate COVID-19 protocol modifications approved by the institutional review boards and research organizations.

IMMUNOPATHOPHYSIOLOGY OF COVID-19 RELATED PULMONARY SYNDROME

Transmitted by droplets, novel coronavirus gains entry *via* angiotensin-converting enzyme 2 receptors located on the epithelial lining of nose, mouth, throat and multiplies within type II epithelial cells within the lung parenchyma[15]. COVID-19 related pulmonary syndrome ranges from mild upper airway symptoms progressing to life-threatening ARDS and related cardiopulmonary failure. Initial COVID-19 pneumonia appearances on histopathology are characterized by alveolar oedema, with patchy and mild inflammatory reaction. Advanced COVID-19 pneumonia can have appearances similar to ARDS characterized by alveolar congestion, oedema, haemorrhage, and end-stage fibrosis. The wide spectrum of COVID-19 syndrome ranges from being asymptomatic to mild upper respiratory tract symptoms to severe viral

pneumonia, ARDS, multiple organ dysfunction and eventual demise. On gross pathology of lung specimens, COVID-19 can induce proliferative as well as exudative lung injury. However, major findings on histopathology comprise a triad of alveolar epithelial cell injury, hyperplasia of type II pneumocytes and hyaline membrane formation.

CLINICAL MANIFESTATIONS OF COVID-19 *VS* BRONCHOGENIC CARCINOMA

Symptoms of COVID-19 related pulmonary syndrome may be similar to deteriorating symptoms encountered during bronchogenic carcinoma progression. Similarities between clinical and imaging findings can pose a major challenge to clinicians in distinguishing COVID-19 super-infection from evolving bronchogenic carcinoma. The above-mentioned entities require very different therapeutic approaches. Immune checkpoint inhibitor therapy which is a more widely used treatment for bronchogenic carcinoma can also induce pneumonitis reported in 2% of its patients and may display similar imaging appearances of COVID-19 related pulmonary syndrome[16]. Interstitial pattern of pneumonitis reported in 4% of patients with epidermal growth factor receptor-mutant bronchogenic carcinoma on treatment with tyrosine kinase inhibitor Osimertinib may have similar radiological patterns of COVID-19.

OVERLAP OF COVID-19 IMAGING FINDINGS AND WHAT IT MEANS FOR PATIENTS WITH BRONCHOGENIC CARCINOMA?

Although consolidation and ground-glass opacification associated with COVID-19 related pulmonary syndrome tends to be more peripheral in location, the radiological changes can mimic treatment induced chemotherapy or radiotherapy and immunotherapy related pneumonitis in bronchogenic carcinoma patients. However, the latter tends to be more confluent and perihilar in location[17]. In situations where clinicians encounter overlap in imaging findings, evaluation of such patients by a multidisciplinary team consisting of oncologists and radiologists should be undertaken with due consideration given for reverse-transcriptase polymerase chain reaction (RT-PCR) testing. Computed tomography (CT) pulmonary angiography represents a valuable imaging investigation for diagnosing pulmonary thromboembolism and for planning further management strategies in patients with COVID-19 and lung cancer related complications[18]. Although the current guidelines do not recommend imaging as the first line investigation for screening of COVID-19 related pneumonia, most countries have adopted diagnostic imaging as an adjunct tool for follow-up of disease progression in these patients.

BRONCHOGENIC CARCINOMA AS A RISK FACTOR FOR COVID-19

Patients with bronchogenic carcinoma have a higher susceptibility to develop severe forms of COVID-19 disease and related complications. Furthermore, patients with associated comorbidities including moderate to severe asthma, chronic obstructive pulmonary disease, cardiovascular disease, diabetes mellitus, chronic kidney disease on dialysis, chronic liver disease, immunosuppression conditions and severe obesity are more at risk of developing severe form of COVID-19 disease. Consequently, patients with bronchogenic carcinoma comprise a high-risk group for COVID-19 pneumonia and related complications. Smoking and immunosuppression induced by steroids are cumulative risk factors for COVID-19 in patients with bronchogenic carcinoma.

A study done at Zhongnan Hospital of Wuhan University, China on 1524 cancer patients admitted to the Department of Radiation and Medical Oncology showed that 0.79% of cancer patients had COVID-19 as against 0.37% of the general population of Wuhan[19]. Findings from a retrospective analysis published in China revealed that patients with cancer, especially advanced bronchogenic carcinoma (28%), are at greater risk of severe disease as compared to non-cancerous patients and the same is confirmed by multiple studies done in this aspect[20].

Smoking increases the risk of complications by up to 1.4-fold for deaths, and by 2.4-fold for mechanical ventilation requirement in patients with COVID-19. At a recent meeting of the "American Association for Cancer Research" annual congress on "COVID-19 and Cancer", data from a global registry on patients with thoracic cancers infected with COVID-19 revealed a 34.6% mortality rate amongst patients with thoracic cancer[21]. In addition, the most common complications reported were pneumonitis (79.6%), ARDS (26.8%), multiple organ dysfunction (7.6%), and sepsis (5.1%)[21]. The proceedings from the session also attributed the cause of death among patients with bronchogenic carcinoma to COVID-19 and not to carcinoma per se, putting them at greater risk of developing complications, irrespective of the stage of cancer.

RISK OF INFLAMMATORY DISORDERS IN PATIENTS WITH BRONCHOGENIC CARCINOMA

Increased inflammation termed as hyperinflammation is common with aging and in cancer patients referred to as "inflammaging". Poor prognosis in cancer patients and worse outcomes in elderly with severe COVID-19 disease are attributed to this hyperinflammatory disorder characterized by vast immune cell mediated activation of cytokines. Immune checkpoint inhibitors can also cause hyperinflammatory response, leading to enhanced production of cytokines from macrophages and T- lymphocytes.

Immune dysregulation in patients with bronchogenic carcinoma may cause hyperinflammation with increased risk of severe COVID-19 disease. Based on a study by Peng *et al*[22], which showed an over-representation of cancer patients and bronchogenic carcinoma in particular within their COVID-19 cohort, the authors have proposed three strategies for cancer patients to circumvent the COVID-19 crisis which include; intensifying surveillance of cancer patients infected with COVID-19, increased use of personal protection equipment by cancer patients, and postponing elective surgery for stable cancer patients from endemic areas of COVID-19. Additionally, patients with bronchogenic carcinoma are prone to emergent complications which might require admission to intensive care units or ventilator support which makes them highly vulnerable for contracting COVID-19. Smoking has also been implicated as an independent risk factor for progression to severe COVID-19 disease.

Findings from a study done by Zhou *et al*[19] at Zhongnan Hospital of Wuhan University, China on 1524 cancer patients admitted to the Department of Radiation and Medical Oncology, revealed that patients with bronchogenic carcinoma harboured a higher risk of COVID-19 compared to the general population regardless of the patient being on anticancer treatment. Also, patients with non-small cell lung cancer (NSCLC) and above the age of sixty have a relatively higher risk of contracting COVID-19[23]. Recommendations were issued by collaboration of the Lung Cancer Study Group, the Chinese Respiratory Oncology Collaboration, Chinese Medical Association and Chinese Thoracic Society regarding management of NSCLC patients during the COVID-19 pandemic. These include intentional postponing of antitumor treatment based on the patient performance status; usage of personal protection equipment for patients with advanced NSCLC; treating advanced NSCLC patients as outpatients whenever possible; promptly identifying symptoms of COVID-19 in patients with NSCLC being masked by antitumor treatment; testing patients for COVID-19 before administering antitumor treatment and tailoring treatment strategies based on subtype of NSCLC and efficacy of treatment[24].

ROLE OF IMAGING IN COVID-19 PATHWAY

RT-PCR is the gold standard investigation for the diagnosis of COVID-19 although, there can be a delay in results of RT-PCR[25]. Here, the role of imaging investigations has been deciphered for rapid screening of patients with suspected COVID-19. Non-contrast CT scan is particularly recommended as intravenous contrast may mask subtle ground-glass opacification as a typical finding in COVID-19. Additional findings include basal consolidations and reverse-halo sign. However, a typical CT finding such as pleural effusions, pulmonary nodules, mediastinal lymphadenopathy, tree-in-bud opacities and cavitation may be encountered in a small minority of patients and should raise concern for atypical pneumonia caused by Mycoplasma or Chlamydia. Halo sign and pneumothorax should suggest an alternative diagnosis

rather than COVID-19[26]. As the imaging findings of COVID-19 are not very specific and may show considerable overlap with other viral/atypical pneumonia, the American College of Radiology (ACR) does not recommend routine use of chest radiography or CT for the screening of COVID-19 in asymptomatic individuals or patients with mild symptoms[27]. However, CT is still recommended for making a diagnosis in symptomatic individuals with a high suspicion of COVID-19 but with a negative RT-PCR result and is being utilized for improving the false negative rate of RT-PCR.

Imaging allows further risk stratification of patients when an initial negative result of RT-PCR requires a risk assessment for high clinical suspicion of COVID-19. The British Society of Thoracic Imaging has provided a template for uniform reporting of chest radiography findings based on classification of patients into four groups which include Non-COVID, COVID normal, COVID indeterminate and COVID classic/probable[28]. Classic findings of COVID-19 on chest radiographs include multiple peripheral air space opacities with bilateral multi-lobar consolidations. However, when CT was used as first form of investigation for diagnosis and screening of COVID-19, the challenges for the radiology departments were mainly infection control. Typical findings of COVID-19 on CT scans include ground glass opacification, basal and predominantly peripheral consolidations which were highly sensitive. However, radiological findings of COVID-19 are relatively less specific. Currently, CT is not being used as a first-line investigation for asymptomatic/mild COVID-19 disease but is reserved for patients with high clinical suspicion of moderate to severe COVID-19 disease but with a negative RT-PCR result[29].

There is no role of 18F-fluorodeoxyglucose (18F-FDG) positron emission tomography/CT (PET/CT) in emergency settings but may be used to monitor disease progression and response to treatment. During acute infections, neutrophils are dependent on anaerobic glycolysis to maintain cellular function which is reflected by a concomitant increase in FDG uptake forming the basis of pattern recognition on PET/CT.

RADIOGRAPHIC FEATURES OF COVID-19

The sensitivity of chest radiographs is relatively less ranging between 30%-70% and typical findings of COVID-19 include multifocal translobar consolidation[30]. Pleural effusions are a relatively rare feature. At initial presentation, total positive rate of RT-PCR from nasopharyngeal swabs has been reported to be 59% due to current limitations in kit performance[31]. The European Radiologists Association recommended using chest radiographs as a first-line investigation for triage of COVID-19 patients based on developed diagnostic algorithms[32].

IMAGING FINDINGS OF COVID-19 PNEUMONIA ON CT

Role of chest CT in the initial diagnosis of COVID-19 related pulmonary syndrome is commendable. Typical imaging findings of COVID-19 pneumonia on chest CT include patchy ground glass opacification in basal and peripheral subpleural distribution with translobar consolidations. Crazy paving pattern is a common finding, whereas reverse-halo sign is rare, but when present is highly specific[33]. CT has a determinant role, not only for early diagnosis of COVID-19, but also for monitoring disease progression and clinical course. Early phase of COVID-19 pneumonia is characterized by ground-glass opacification in peripheral and basal distribution, crazy paving pattern, and traction bronchiectasis. Advanced phase of COVID-19 pneumonia is characterized by progressive transition to consolidation, irregular interlobular septal thickening with extensive lung nodules, and ARDS. Progressive stage of bronchogenic carcinoma or concomitant pneumonia in bronchogenic carcinoma patients may have similar overlapping CT findings of COVID-19 related pulmonary syndrome[34].

An expert consensus statement on 'Reporting Chest Computed Tomography Findings Related to COVID-19' has been published by the Radiological Society of North America which categorizes COVID-19 pneumonia based on CT findings into negative for pneumonia, atypical appearances, indeterminate, and typical appearances [35]. CT findings specific for COVID-19 pneumonia include bilateral ground-glass opacification with interlobular septal thickening (crazy paving appearance) predominantly in basal and peripheral distribution with reverse halo sign. CT findings of COVID-19 based on the duration of symptoms include—early stage constituting 0-4 d

after onset of respiratory symptoms. Findings during this stage include normal CT scans in 50% of patients with the rest demonstrating focal subpleural ground glass opacification, predominantly in lower lobes. However, typical CT findings are rarely observed during this phase. Progressive stage of COVID-19 constitutes 5-8 d after symptom onset and is characterized by multifocal and bilateral ground glass opacification on CT scans in approx. The 50%-75% of patients, demonstrating progression to crazy paving appearances. Peak stage of COVID-19 constitutes 9-13 d after symptom onset characterized by crazy paving pattern with multifocal and translobar consolidations with air bronchograms and features of ARDS[36].

DIAGNOSIS AND STAGING OF BRONCHOGENIC CARCINOMA DURING TIMES OF COVID-19

General recommendations include minimize aerosol generation and limiting COVID-19 transmission by giving preference to image-guided transthoracic biopsies for diagnosing bronchogenic carcinoma over transbronchial approaches[37]. Giving preference to non-invasive mediastinal staging *via* imaging on PET/CT and CT and if not feasible preferring mediastinoscopy over bronchoscopy. Endobronchial ultrasound (EBUS) as an investigative tool for nodal staging of stage III disease in absence of radiographically apparent mediastinum may not be required, and for stage III disease with radiographically apparent nodal disease of the mediastinum, no further confirmation with EBUS is required[38].

COVID-19 AND BRONCHOGENIC CARCINOMA

Cancer survivors and patients represent a vulnerable population for COVID-19 syndrome which can further complicate respiratory and cardiovascular comorbidities they possess. Prevalence of COVID-19 in cancer patients is approx. 1%-6% as compared to their non-cancerous counterparts[39]. And, patients with bronchogenic carcinoma have the highest risk of unfavourable outcomes and complications of COVID-19. Though reasons for this selective affinity of COVID-19 patients is not fully elucidated, lower immune response in cancer patients secondary to treatment regimens may be the cause. Other causes include advanced age of patients and smoking as a risk factor which is prevalent in patients with bronchogenic carcinoma. Additionally, pre-existing cardiovascular and pulmonary comorbidities in patients with bronchogenic carcinoma make them highly vulnerable to COVID-19 and can further complicate the course of the disease. The cumulative risk factors identified in patients with bronchogenic carcinoma complicate the pathway of COVID-19 related pulmonary syndrome in them resulting in an unfavourable outcome.

SCREENING OF BRONCHOGENIC CARCINOMA DURING TIMES OF COVID-19

Streamlining resources by healthcare stakeholders for screening of bronchogenic carcinoma and evaluation of patients with incidentally detected lung nodules are related scenarios which are currently in focus during these uncertain times of a pandemic. National Comprehensive Cancer Network, the United States Preventive Services Taskforce, and CHEST (the American College of Chest Physicians) have issued guidelines for screening of patients to rule out bronchogenic carcinoma and have recommended annual low-dose chest CT to be undertaken in high-risk individuals, wherein the benefits of screening are likely to outweigh the risks[40]. The ACR, the British Thoracic Society, the Fleischner Society, and CHEST have all published guidelines based on recommendations for evaluating incidentally detected lung nodules, in which larger lung nodules are screened based on the estimated probability of malignancy (pCA) and the benefit of additional testing. Smaller nodules in comparison are screened based on the size and attenuation features of the nodule as well as the presence of risk factors for bronchogenic carcinoma[41].

IMPLICATION OF COVID-19 ON SCREENING OF BRONCHOGENIC CARCINOMA

Attempts at screening for bronchogenic carcinoma in patients should be delayed until the curve of the pandemic flattens and risks involved with transmission of COVID-19 becomes near minimal. The reasons for the same include increased risk of exposure of patients to COVID-19. Incidental lung findings in asymptomatic individuals exposed to COVID-19 increases the false positive rate and may expose these patients to unnecessary procedures thus increasing the risk further. Thus, by delaying screening for cancer in the current pandemic, resources can be streamlined for managing patients with moderate to severe COVID-19 disease.

IMPACT OF COVID-19 ON PATIENTS WITH BRONCHOGENIC CARCINOMA

The COVID-19 pandemic has strained the healthcare system and drained the resources worldwide due to high transmission rate causing significant mortality and morbidity. Super-infection with COVID-19 in patients with bronchogenic carcinoma increases the risk of complications with further delay in treatment of bronchogenic carcinoma resulting in poor outcomes and mortality. The impact of COVID-19 on patients with bronchogenic carcinoma is multi-faceted and management should be focused in improving negative outcomes. The implications of COVID-19 pandemic in bronchogenic carcinoma management requires multidisciplinary teams to strike a balance between treating bronchogenic carcinoma and simultaneous treatment of COVID-19 disease and related complications.

WEIGHING BENEFITS *VS* RISKS IN THE MANAGEMENT OF BRONCHOGENIC CARCINOMA

The current pandemic has forced all healthcare stakeholders to prioritize per value resources and reorganize therapeutic strategies for timely management of patients with COVID-19 related pulmonary syndrome. Since the beginning of the COVID-19 pandemic, the oncology community has been under tremendous strain in managing patients with bronchogenic carcinoma that requires a balance in treating patients, without losing them to COVID-19. Organizations worldwide such as the European Society of Medical Oncology (ESMO), have worked to implement preventative measures and spreading knowledge about the additional risks that cancer patients are prone to during the COVID-19 pandemic[42]. Oncologists have faced the constant dilemma of whether to offer or postpone treatment to patients during this COVID-19 pandemic. Consideration of bronchogenic carcinoma treatment during COVID-19 times requires careful individual assessment as chemotherapy administered may cause transient immune suppression. In addition to this, immunotherapy with immune checkpoint inhibitor therapy may trigger chronic inflammatory changes within the lung parenchyma causing pneumonitis like picture, with resultant poor outcome. However, oncologists currently should carefully weigh the benefits of treating patients with bronchogenic carcinoma *vs* the risk of getting infected with COVID-19, as delay in treatment may also have a negative effect on the prognosis of bronchogenic carcinoma causing its progression.

IMPLICATION OF COVID-19 ON PULMONARY MEDICINE

Pulmonologists and pulmonary oncologists comprise front-line specialists dealing with the diagnosis and management of patients with bronchogenic carcinoma and COVID-19 related syndrome. It is very crucial for delineating the differential diagnosis of bronchogenic carcinoma and COVID-19, as there is an overlap in symptomatology of the two entities. The delineation and differentiation are based on rapid progression of symptoms, positivity of RT-PCR results to COVID-19, fresh appearance of a patchy infiltrate that is distinct from the baseline pattern of bronchogenic carcinoma initially encountered on imaging.

IMPLICATION OF COVID-19 ON RADIOLOGY

COVID-19 pulmonary syndrome, bronchogenic carcinoma, chronic obstructive pulmonary disease, pulmonary arterial thromboembolism, lung metastases, radiation pneumonitis, radiation fibrosis and lobar pneumonia are some of the pulmonary conditions for which radiological diagnostic modalities such as chest radiographs, CT and PET/CT form the cornerstone of management and follow up. However, sociocultural approaches and published guidelines for imaging of suspected or confirmed cases of COVID-19 differ for these diagnostic modalities with a temporal and regional variation. Peripheral/subpleural ground-glass opacification with a predilection to bilateral posterior lung segments, multifocal consolidations, crazy paving pattern, vascular dilatation with interlobular septal thickening, traction bronchiectasis and reverse halo sign are some of the typical features of COVID-19 encountered on CT[43]. Whether it is COVID-19 or bronchogenic carcinoma, radiologists play an important role for the diagnosis and monitoring of disease progression in these patients. Radiologists should be familiar with the radiological patterns of bronchogenic carcinoma and the appearances of COVID-19 related pulmonary syndrome as imaging has a critical role in diagnosing, monitoring disease progression and prognostic follow up. Utmost care should be taken while encountering findings of COVID-19 in patients with bronchogenic carcinoma, as this may falsely upstage the grade of bronchogenic carcinoma or be reported as progressive disease which may have implications on future management.

MANAGING PATIENTS WITH DIFFERENT SCENARIOS

Choice of chemotherapy, targeted therapy, immunotherapy and supportive care must be tailored based on tumour characteristics and patient comorbidities, considering the risk of COVID-19 super-infection in these patients. Prioritizing the choice of treatment is dependent on active COVID-19 cases in the community and the availability of resources. Prima facie principle in the management of patients with bronchogenic carcinoma is to provide timely enrolment in treatment without unjustified delay for better prognosis. Patients with bronchogenic carcinoma who are asymptomatic or showing features of minimal progression may be monitored at a 4-8 wk interval, and radiotherapy may be offered to patients with local disease progression[44]. However, progressive disease in bronchogenic carcinoma and immune checkpoint inhibitor therapy induced pneumonitis should be differentiated from COVID-19 related pulmonary syndrome. The treatment of COVID-19 as priority and subsequent immune checkpoint inhibitor therapy should be continued when a patient with bronchogenic carcinoma is diagnosed with COVID-19 while on treatment with tyrosine kinase inhibitors[45].

DIAGNOSTIC STRATEGIES FOR PATIENTS WITH BRONCHOGENIC CARCINOMA

During these unprecedented times of COVID-19 pandemic, using the least invasive technique holds the key for diagnosing bronchogenic carcinoma in patients. Aerosol-generating procedures such as bronchoscopy should be avoided whenever feasible [46]. A statement issued by the American Association for Bronchology and Interventional Pulmonology regarding the effective use of bronchoscopy in patients with suspected or confirmed cases of COVID-19 also applies to patients with bronchogenic carcinoma[47]. Salient features of the consensus statement include elective bronchoscopy procedures for bronchial and pulmonary mass lesions, mild-to-moderate tracheal stenosis and mediastinal or hilar lymphadenopathy should be postponed until full recovery from COVID-19. For serious emergent conditions such as malignant severe airway stenosis, massive haemoptysis, malignant cause of endobronchial obstruction and rapidly progressing malignancy, bronchoscopy as a lifesaving intervention for urgent or emergent reasons should be considered with all precautionary measures[47]. Categorization of all procedures, including transthoracic needle biopsies have been put into emergent, urgent and elective, based on priority and risk status to the patient, have been done by the Society of Interventional Radiology[48].

Early identification of COVID-19 and differential diagnosis in patients with bronchogenic carcinoma

Symptoms of patients with bronchogenic carcinoma is due to the tumour itself and may also include cough, dyspnoea and occasional fever. Also, treatment related adverse events caused by immunotherapy, radiotherapy and chemotherapy may also be encountered in these patients. Further, different patterns of bronchogenic carcinoma presenting with varied clinical manifestations have been noted on chest CT. Early recognition of patterns for differentiating COVID-19 from bronchogenic carcinoma in patients is extremely important, for elucidating the differential diagnosis and for planning of management strategies.

Further differential diagnoses

Patients with an epidemiological history who fulfil any two of the clinical criteria or patients with no clear epidemiological history but having fulfilled three of the clinical criteria are considered as suspicious patients based on the diagnostic criteria of COVID-19. It is imperative to distinguish patients with clinical characteristics of bronchogenic carcinoma progression from those with COVID-19. Further differential diagnoses to be considered include: (1) Pulmonary infections which may be atypical pneumonia caused by *Chlamydia*, *Mycoplasma*, *Pneumocystis jirovecii*, *Legionella* or non-COVID-19 viral pneumonia caused by adenovirus, influenza virus, cytomegalovirus and respiratory syncytial virus; (2) Radiation pneumonitis which occurs 1-3 mo after undergoing radiotherapy in patients with bronchogenic carcinoma[49]. Clinical symptoms may include dry cough associated with fever and decreased white blood cell count. Findings on chest CT in radiation pneumonitis are mostly nonspecific and may include ground-glass opacities opacification with patchy infiltrates. Differentiation from COVID-19 related pulmonary syndrome is difficult; (3) Tumour progression in bronchogenic carcinoma may result in lymphangitis carcinomatosa, pneumonic-type lung carcinoma, and obstructive pneumonitis which may present with fever and/or worsening respiratory symptoms; (4) Clinical characteristics of immune checkpoint inhibitor-related pneumonitis include fever, worsening of cough and dyspnoea, ground glass opacification and patchy infiltrates on the chest CT; and (5) Miscellaneous differential diagnoses include cardiac insufficiency and pulmonary thromboembolism.

CURRENT IMAGING PERSPECTIVES

Imaging plays a crucial role in the diagnosis, management and follow-up of immunotherapy related complications of bronchogenic carcinoma[50]. Centers for Disease Control and Prevention (CDC) has provided recommendations which were endorsed by the ACR, for safeguarding and prioritizing healthcare resources during the COVID-19 pandemic[51]. The recommendations state that imaging for stable patients, including elective diagnostic or image-guided procedures in non-critical patients which have a low priority should be advertently postponed and rescheduled until the curve of the pandemic flattens.

Pulmonary oncologists and radiologists should collaborate for rescheduling and reviewing chest CT examination, based on the symptomology and prognostic value of such investigation during times of COVID-19 pandemic (low priority). A low-dose CT protocol for screening of bronchogenic carcinoma should be temporarily withheld or advertently postponed considering the risk-benefit ratio of such a procedure in the current situation. Follow-up imaging for bronchogenic carcinoma during the course of treatment in the first 6 mo or in case of bronchogenic carcinoma with progression at any point in time should be considered (high priority). Recommendations suggest priority access to diagnostic imaging in patients with clinical picture suspicious for bronchogenic carcinoma (high priority) (Figure 1).

BRONCHOGENIC CARCINOMA SCREENING: BASELINE AND ANNUAL

When an individual who satisfies the eligibility criteria is referred for screening of bronchogenic carcinoma, initiation of screening should be delayed during the COVID-19 pandemic based on the consensus statement consistent with CDC guidance[52]. If an individual who satisfies the eligibility criteria is due for a repeat annual chest CT, annual screening examination should be delayed too. Factors that may influence this

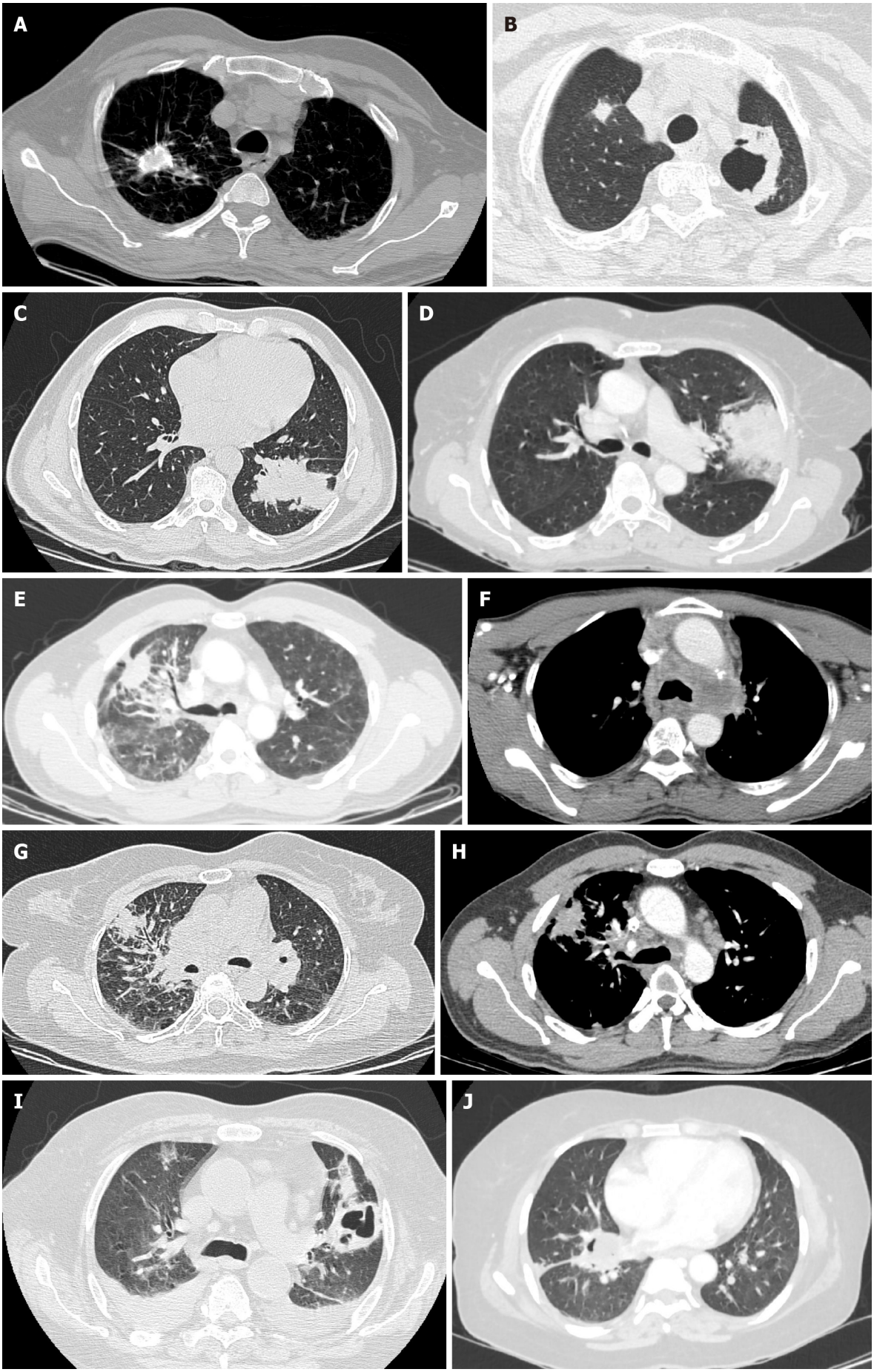

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Figure 1 Axial high-resolution computed tomography images. A: Axial high-resolution computed tomography (CT) chest image demonstrating small cell carcinoma of the right lung with dystrophic calcifications; B: Axial high-resolution CT chest image demonstrating central cavitatory squamous cell carcinoma of the left lung. Note the metastatic lesion in the right lung; C: Axial high-resolution CT chest image demonstrating large solid mass lesion with lobulated margins in a case of adenocarcinoma of the left lung; D: Axial high-resolution CT image of chest demonstrating extensive peripheral consolidation with numerous air bronchograms in a case of bronchoalveolar carcinoma of left lung; E: Axial high-resolution CT chest image demonstrating mass lesion with surrounding ground glass component representing lepidic tumor growth in a case of adenocarcinoma of the right lung; F: Axial contrast-enhanced CT image at the level of mediastinum demonstrating left hilar mass lesion with mediastinal invasion in a case of adenocarcinoma of the lung; G: Axial high-resolution CT chest image demonstrating primary bronchogenic carcinoma in the right lung with nodular and irregular interlobular septal thickening consistent with features of lymphangitis carcinomatosa; H: Axial contrast-enhanced CT chest image demonstrating sign of short burrs and spinous processes of tumor margins in a case of squamous cell carcinoma of the right lung. Note the tapered extension of the lesion to pleura and adjacent pleural retraction; I: Axial high-resolution CT chest image demonstrating peripheral cavitatory squamous cell carcinoma of the left lung. Note bilateral pleural effusions; J: Axial high-resolution CT image of chest demonstrating mass lesion invading the oblique fissure of the right lung in a case of non-small cell lung carcinoma.

decision include availability of resources, COVID-19 penetrance in the community, and comorbid conditions.

SURVEILLANCE OF A PREVIOUSLY DETECTED LUNG NODULE

When an individual who satisfies the eligibility criteria is due now for a surveillance chest CT for an incidentally detected lung nodule, delaying the surveillance CT scan for approximately 3 mo to 6 mo is acceptable based on the consensus statement consistent with CDC guidance[53].

EVALUATION OF INTERMEDIATE AND HIGH-RISK LUNG NODULES

When an individual who satisfies the eligibility criteria is due now for an imaging guided biopsy, delaying the procedure and re-evaluation of the patient with chest CT in approximately 3 mo to 6 mo is acceptable too based on the consensus statement consistent with CDC guidance[54]. However, pre-COVID-19 recommendations suggest further evaluation with PET/CT and/or biopsy for the patient with intermediate and high-risk lung nodules.

MANAGEMENT OF STAGE I NSCLC

When an individual who satisfies the eligibility criteria for surgical treatment of clinical stage I NSCLC, surgery may be deferred based on the consensus statement consistent with CDC guidance after taking into consideration the size, growth rate and 18F-FDG PET avidity of the malignant lesion[55]. Nevertheless, the clinical scenario needs to be discussed at a multidisciplinary tumour board meeting for delaying treatment in an indolent/early-stage bronchogenic carcinoma during times of the COVID-19 pandemic.

PRIORITIES FOR IMAGING OF BRONCHOGENIC CARCINOMA DURING THE COVID-19 PANDEMIC

Criteria for high priority imaging include: (1) Patients with clinically significant respiratory symptoms with a history of bronchogenic carcinoma; (2) Bronchogenic carcinoma patients with fresh appearance of respiratory symptoms such as dyspnoea, cough and fever; (3) Patients with suspected bronchogenic carcinoma of unknown stage or stage II, III, IV for standard staging work-up; (4) Patients with mass lesion / nodules suspicious for bronchogenic carcinoma stage III/IV for biopsy; and (5) Patients with bronchogenic carcinoma for suspicion of carcinoma progression at any point of time or for evaluation of response during the first 6 mo of treatment.

Criteria for medium priority imaging include: (1) Patients with early bronchogenic carcinoma for determining the stage; (2) Patients with mass lesion / nodules suspicious for bronchogenic carcinoma of unknown stage I/II for biopsy; (3) Patients on treatment for bronchogenic carcinoma with fresh appearance of clinical symptoms; (4)

Stable patients of bronchogenic carcinoma for evaluation of response beyond 6 mo of treatment; (5) Follow-up imaging for patients of bronchogenic carcinoma having underwent radical treatment with high/intermediate risk of relapse; (6) Follow-up imaging for solid nodules with volume of 50-500 mm^3, partly solid nodule with a non-solid component of size ≥ 8 mm in diameter or a pleural-based solid nodule of size 5-10 mm in diameter; and (7) Bronchogenic carcinoma patients with Volume Doubling Time (VDT) ranging 400-600 d.

Criteria for low priority imaging include: (1) Follow-up imaging for patients of bronchogenic carcinoma having underwent radical treatment more than a year ago with high/intermediate risk of relapse; (2) Follow-up imaging for patients of bronchogenic carcinoma having underwent radical treatment with low risk of relapse; (3) Follow-up imaging for solid nodules with benign morphology, nodules with volume of < 50 mm^3, partly solid nodule with a non-solid component of size < 8 mm in diameter, or a pleural-based solid nodule of size < 5 mm in diameter; (4) Bronchogenic carcinoma patients with VDT > 600 d; and (5) Low-dose CT screening of patients for bronchogenic carcinoma screening can be deferred until the current COVID-19 pandemic resolves which is unlikely to affect the overall survival rate (Table 1).

GUIDING PRINCIPLES FOR TREATMENT OF PATIENTS WITH BRONCHOGENIC CARCINOMA

The most significant goal of bronchogenic carcinoma management during these challenging times of the pandemic is to minimize the risk of exposing patients and staff to COVID-19 whilst still managing all life-threatening events related to bronchogenic carcinoma. This priority can be achieved by limiting outpatient visits to the hospital, and extensive testing for COVID-19 in patients undergoing invasive procedure or systemic chemotherapy and immunotherapy.

Early-stage bronchogenic carcinoma

Ablative radiotherapy and surgical resection are the treatment approaches for patients with stage I/II/resectable stage III NSCLC subtype of bronchogenic carcinoma[56]. However, the principles for surgical resection of bronchogenic carcinoma remain unaffected during this COVID-19 pandemic. Recommendations from CDC and guidelines from professional societies calls for rescheduling of elective surgeries during the current impending pandemic[57]. Recommendations form the American Society of Clinical Oncology state that patients with cancer and treating clinicians should make individual considerations based on risks of delaying surgical resection in these patients[58]. Also, rescheduling of surgical resection for patients with a recent diagnosis of early-stage bronchogenic carcinoma is recommended, as super-infection with COVID-19 may have poor prognostic outcomes.

The European Association of Medical Oncology has recommended considering surgical resection as a priority in the management of early NSCLC and delays in surgery not to exceed 6-8 wk[59]. Recommendations based on the phase of impending COVID-19 pandemic and availability of resources by healthcare institutions, the American College of Surgeons has recently published COVID-19 triage guidelines focusing on surgical care in elective cases[60]. Based on recommendations in semi-urgent settings, surgical intervention is reserved for patients whose survivorship is likely to be compromised when surgical resection is not performed within the next 3 mo. Some of them could be node-positive bronchogenic carcinoma, bronchogenic carcinoma greater than 2 cm in the greatest dimension, and solid or predominantly solid (> 50%) bronchogenic carcinoma[61]. However, surgical resection should be deferred for indolent cases of bronchogenic carcinoma, solid nodules less than 2 cm and ground-glass nodules. Based on recommendations in urgent settings, surgical intervention is reserved for patients likely to have poor prognosis if a surgical intervention is not performed within the next few days, such as surgical complications and tumour associated super-infection[61]. Additionally, stereotactic ablative radiotherapy and neoadjuvant therapy are recommended for these patients.

Surgical resection should be deferred for 2-3 wk in patients with bronchogenic carcinoma who test positive for COVID-19 (Figure 2). When a surgical intervention is deemed inevitable, a specialized negative pressure operating theatre with proper usage of personal protective equipment and a negative-pressure isolation room for postoperative care are recommended[60]. If a surgical procedure is rescheduled, it is advisable for bronchogenic patients to be retested for COVID-19 before the procedure. Reasons for this approach include chemotherapy-induced immunosuppression, which

Table 1 Imaging priorities for bronchogenic carcinoma

High priority	Medium priority	Low priority
Patients with significant respiratory symptoms and/or cancer-related or treatment-related symptoms. In patients with new onset respiratory symptoms such as dyspnoea, cough with or without fever, a CT scan is recommended	Follow-up imaging for high/intermediate risk of relapse in a year after completion of radical treatment	Follow-up imaging for high/intermediate risk of relapse more than 1 yr after completion of radical treatment
Standard staging work-up for suspected invasive cancer of unknown stage or stage II/III/IV	Standard staging work-up for early lung cancer (stage I)	Follow-up imaging after radical treatment in low-risk of relapse scenario
Biopsies for suspicious nodules or mass for suspected invasive cancer or stage III/IV	Biopsies for suspicious nodules or mass for suspected invasive cancer of unknown stage or stage I/II	
Evaluation of active treatment response in the first 6 mo of treatment or for suspicion of tumour progression at any point of time	Evaluation of active treatment response beyond 6 mo of treatment if stable/ controlled situation	
	Follow-up of nodules of incidental finding with either: (1) Partially solid nodule with a non-solid component of ≥ 8 mm in size; (2) Known VDT 400 d to 600 d; (3) Solid nodule 50 mm^3 to 500 mm^3; and (4) Pleural-based solid nodule 5 mm to 10 mm in size	Follow-up of nodules of incidental finding with either: (1) Partially solid nodule with a non-solid component of < 8 mm in size; (2) Known VDT > 600 d; (3) Solid nodule < 50 mm^3; (4) Pleural-based solid nodule < 5 mm in size; (5) Non-solid nodule < 8 mm in size; and (6) Benign morphology
Pre-planned imaging evaluation per clinical trial protocol		Lung cancer screening can be deferred until the COVID-19 pandemic resolves — it is reasonable for patients in the general population to defer screening low-dose CT, a deferral that is not likely to have an impact on overall survival

COVID-19: Coronavirus disease 2019; CT: Computed tomography; VDT: Volume doubling time.

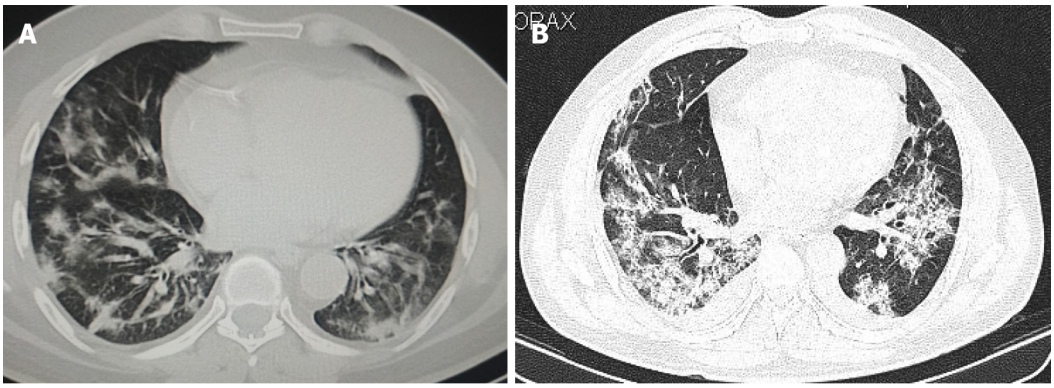


Figure 2 Axial high-resolution computed tomography images of chest. A: Axial high-resolution computed tomography (CT) image of chest on day 5 after symptom onset demonstrating peripheral predominant consolidation pattern with areas of ground glass opacification in bilateral lower lobes in a patient with coronavirus disease 2019 pneumonia; B: Axial high-resolution CT image of chest on day 9 after symptom onset demonstrating extensive consolidation predominantly in basal segments of bilateral lower lobes in a patient with coronavirus disease 2019 related pulmonary syndrome. Note the bilateral pleural effusions which is an atypical finding in coronavirus disease 2019.

might increase the risk of exposure to COVID-19 and severe pulmonary complications henceforth and to help prevent the risk of contracting COVID-19 during frequent trips to the hospital for chemotherapy cycles. Recommendations suggest using neoadjuvant therapy can achieve better prognosis in patients with bronchogenic carcinoma in whom surgical intervention has been postponed due to the above-mentioned reasons [61]. Home management including telemedicine limiting outpatient visits are recommended for cancer patients during this COVID-19 pandemic[62].

Stereotactic ablative radiotherapy and stereotactic body radiotherapy (SBRT) are non-invasive treatment strategies in the management of early stage (< 5 cm) node-negative NSCLC[61,62]. SBRT is a well-established, highly effective treatment modality with minimal risk and good local growth control of tumour[63]. SBRT involves delivery of single fraction of 24-34 Gy for peripheral tumours of size less than

2 cm or delivery of 5 to 10 fractions of 50-70 Gy for central tumours. Considerations are to be made as to delay treatment for small sized slow-growing tumours or to deliver treatment immediately for larger tumours showing rapid growth in patients with bronchogenic carcinoma in whom SBRT is deemed appropriate. However, during the COVID-19 pandemic maximal use of single fraction treatment with shortening of SBRT fractionation schemes are to be implemented. Another promising treatment modality is brachytherapy which involves insertion of a radioactive source within the tumour causing less radiation dose to surrounding tissues adjacent to the tumour, and is primarily utilized for small endobronchial obstructive lesions, recurrent, or early-stage bronchogenic carcinoma. Since brachytherapy increases the risk of exposure to patients and health care providers, it requires multidisciplinary coordination in an enclosed operating theatre with a brachytherapy suite and related personal protective equipment. Preferentially, during the COVID-19 pandemic, considering external beam radiotherapy for treatment of NSCLC patients and avoiding all brachytherapy procedures is recommended[64].

Locally advanced bronchogenic carcinoma

Local resection, systemic chemotherapy and radiotherapy are treatment modalities currently used in the treatment of locally advanced bronchogenic carcinoma, although, concurrent chemoradiotherapy consisting of platinum-based chemotherapy agents with radiotherapy-based delivery of 30 fractions of 60 Gy can also be administered for patients with stage III NSCLC. For curative treatment of bronchogenic carcinoma, factors to be taken into consideration include the risk of contracting COVID-19, adverse effects related to treatment, and availability of resources. Systemic therapies with shorter treatment course and reduced risk of myelosuppression are recommended[65].

PREVENTION OF COVID-19 IN PATIENTS WITH BRONCHOGENIC CARCINOMA

Recommendations for cancer therapy include tailored treatment for patients with bronchogenic carcinoma, including NSCLC after weighing the risk-benefit ratio during times of COVID-19 pandemic. However, the prescribed recommendations are aimed at reducing the risk of COVID-19 transmission. Since NSCLC patients are prone to be reactive during the course of treatment even if drug-induced pneumonitis is suspected, it is necessary to rule out COVID-19 related pulmonary syndrome[66]. In light of the current COVID-19 pandemic, low-dose CT of chest which was traditionally performed for bronchogenic carcinoma screening should be rescheduled especially in patients with underlying cardiopulmonary abnormalities which can put them at increased risks for mortality from COVID-19 pneumonia. Additionally, higher infection rates of COVID-19 in patients with bronchogenic carcinoma is partly attributed to the need to visit medical institutions, especially for performing diagnostic chest imaging, which significantly increases the risk of contact with infected patients and medical personnel alike.

INCIDENTAL DETECTION OF BRONCHOGENIC CARCINOMA ON CT

COVID-19 has caused considerable disruption in screening of bronchogenic carcinoma. During the pandemic, there were fewer new patients screened, more patients were apprehensive to visit the healthcare system, and an increased proportion of nodules were suspected to be malignant. However, there were rare instances encountered where incidental detection of bronchogenic carcinoma was made possible while performing routine chest CT scans in patients with COVID-19 related pulmonary syndrome. The number of newly detected cases with asymptomatic focal changes in lungs have significantly increased during the COVID-19 pandemic. Identifying bronchogenic carcinoma early on routine CT scans undertaken during the pandemic may provide a critical advantage to patients, especially those with heightened risk. This review can provide a framework for future decisions amid the ongoing COVID-19 pandemic.

COVID-19 AND ITS IMPACT ON BRONCHOGENIC CARCINOMA RESEARCH

Clinical research on bronchogenic carcinoma during the current pandemic has been affected due to implementation of strict measures for limitation of COVID-19 spread, long periods of quarantine, lack of resources and varying work shifts. Major research organizations and committees worldwide have implemented decisions of temporarily halting or even permanently stopping clinical research trials for protocol adherence and for preserving the accuracy of endpoint evaluation. Currently clinical trials focused on the research and development of COVID-19 vaccine are the need of the hour, though there is no real surety that a vaccine might be our only ray of hope in these critical times. It is difficult to justify exposing patients to the risk of COVID-19 in clinical trials with a placebo arm, and hence should be suspended for the time being [67].

FUTURE PERSPECTIVES: ROLE OF ARTIFICIAL INTELLIGENCE AND DEEP LEARNING

During pandemics like the current COVID-19, benefits of artificial intelligence (AI) primarily relate to pattern recognition based on imaging findings and the potential of AI for high throughput analysis[68]. Parallel AI processing of images may facilitate triage of patients in institutions located in regions with limited resources and high disease prevalence. Subtle changes across imaging findings on serial CTs may be overlooked, due to human error. However, recent techniques such as AI and computed aided diagnosis may help quantify these changes with precision, and implementation of trend analysis based on baseline trends may help track disease progression in individuals and across populations[69]. The central theme of AI relies on the plasticity of deep learning networks and in adapting to imaging-based recognition of varying disease patterns. Early AI investigators have laid the foundation for detailed exploration of deep learning algorithms for pattern recognition and quantification of disease severity, and for rapid screening of individuals in situations like the current COVID-19 pandemic.

Literature reviews of published earlier studies have reported feasibility of developing AI and deep learning algorithms based on quantitative opacity measurements and lung segmentation models with high sensitivities and specificities, having values reportedly reaching the 90th percentile range. The drawback and limitations of AI and deep learning modules relate to its inherent image-based framework and inability to determine select imaging-based features responsible for determination of output and eventual analysis of the subtype in patients with bronchogenic carcinoma[70].

CONCLUSION

The COVID-19 pandemic has caused a significant health care crisis and has strained the health care systems of developed and developing nations across the world. Recent ESMO recommendations provide social responsibilities for pulmonary oncologists which include providing timely management for cancer patients, while taking necessary precautions to protect patients and themselves from COVID-19 and providing for fair distribution of resources during the pandemic. Treatment decisions should be aimed at striking a balance between risk *vs* benefit ratio which require multidisciplinary discussions between patients and caregivers. In resource starved settings such as the current COVID-19 pandemic, cancer patients who commit to treatment also have a duty to follow safe health practices and to undergo self-isolation. Risk stratification of bronchogenic carcinoma patients and priority imaging may be applied for optimal use of resources. For the first time in the era of modern medicine, all of humankind is facing the same threat considering the contagiousness of the coronavirus and the need to reduce nosocomial outbreaks which determine poor prognosis and detrimental outcomes in patients with bronchogenic carcinoma. This also gives us ample opportunity to change our research approach to one that involves better understanding of disease manifestations; and to further explore and promote the applications of chest CT for the safe management of patients and health personnel in the setting of pandemics like the present or any which we might encounter in the near future. It has been over 12 mo since we first heard of the COVID-19 pandemic

and while promising developments are being made on the clinical trials front and in the management of patients with bronchogenic carcinoma, there is no certainty of events falling into normalcy in the immediate future. Research findings need to be shared amongst countries to provide best health care to humankind during these uncertain times. Modified and newer therapeutic strategies for patients with bronchogenic carcinoma have been adopted by oncologists around the world for providing uncompromised care within the accepted standards and new guidelines.

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MINIREVIEWS

Chemotherapy-induced neurotoxicity in the treatment of gynecological cancers: State of art and an innovative approach for prevention

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Abstract

Chemotherapy-induced peripheral neuropathy (CIPN) is a common side effect that occurs in 20% of ovarian cancer patients treated with the combination of carboplatin/paclitaxel (CP). This toxicity is directly correlated with the dose of paclitaxel administered. Several studies have investigated whether different formulations of taxane can induce this side effect at a lower rate, but, unfortunately, no significant improvement was obtained. CIPN can be disabling in the daily lives of patients and can cause dose reduction or early termination of the treatment. Neuropathy can last for months and even years after its onset. Moreover, patients responsive to CP treatment are candidates for a reintroduction of the same drugs when disease relapse occurs, and residual neuropathy can affect the continuation of treatment. There are no approved drugs that mitigate or prevent the onset of CIPN. In this review, we summarize the evidence regarding the incidence of CIPN with different taxane formulations, regimen schedules and prevention systems. In particular, the Hilotherm® Chemo care device is a regional cooling system that lowers the temperature of the hands and feet to reduce the flow of chemotherapy into the capillaries. We used hilotherapy during chemotherapy infusion to prevent the onset of CIPN. Updated data from 44 ovarian cancer patients treated with 6 cycle of CP show that hilotherapy was well tolerated; only two patients (4.5%) stopped hilotherapy because of cold intolerance, and only one patient (2.2%) experienced grade ≥ 2 CIPN.

Key Words: Peripheral neuropathy; Ovarian cancer; Paclitaxel; Chemotherapy-induced peripheral neuropathy prevention; Hilotherapy

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Core Tip: The association of carboplatin and paclitaxel is the standard chemotherapy regimen for ovarian cancer women. One of the most common and disabling side effect of this treatment is the peripheral neuropathy caused by paclitaxel, it can occur in 20% of patients and can last months and even years after the end of chemotherapy. In this review we collected the evidence from several studies with different schedules or formulation of paclitaxel and the systems of prevention and propose a mechanical way of prevention adopted in our center through the use of the cooling system of the hilotherm device.

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INTRODUCTION

Carboplatin/paclitaxel (CP) is the chemotherapy of choice for ovarian cancer, in neo/adjuvant, first-line and in platinum-sensitive recurrence, and moreover, in advance stage a reintroduction of paclitaxel (PTX) monotherapy can be considered[1]. A significant proportion of recurring patients, with a platinum-free interval longer than 6-12 mo, are classified as platinum-sensitive and are candidates for retreatment with the same drugs used in first-line therapy[2]. Chemotherapy-induced peripheral neuropathy (CIPN) is one of the most life altering chemotherapy side effects[3,4] and it is frequently related to taxane treatment. The prevalence of CIPN ranges from 11% to over 87%[5]. In an online survey, 78% of 1360 women with ovarian cancer experienced CIPN 2.3 years after completing chemotherapy[6]. CIPN leads to peripheral nerve dysfunction or damage of varying intensity, duration and permanence. Symptoms range from acute transient paraesthesia or chronic pain to permanent peripheral nerve damage[4]. CIPN can occurs days after chemotherapy and gradually progresses to cumulative doses. Symptoms typically present as a "glove and stocking" neuropathy, with tingling, loss of dexterity in the fingers, hypoesthesia of the feet, and paraesthesia in the limbs. In addition, skin burns, electric shocks, as well as altered perception of pain to touch or heat may be felt[7-10]. In the worst case, sensory perception can be completely lost. CIPN can also manifest with motor symptoms with distal weakness, gait and balance disturbances, and impaired movements. Daily physical functioning can be impaired such as firmly grasping objects, unfastening jacket buttons, climbing steps or driving, it affects patient's the quality of life (QoL)[11,12]. Health-related QoL can be impaired[6,11,13] whit psychological consequences[14] such as major depression[15].

Due to this adverse event, treatment must unfortunately be interrupted or continued at a reduced dose, limiting the effectiveness of chemotherapy. To date, none of the numerous drugs tested to prevent or relieve the symptoms of CIPN has obtained evidence of efficacy. Antidepressants (such as nortriptyline), duloxetine, gabapentin, baclofen-containing gel, amitriptyline, ketamine, lidocaine, tramadol, tapentadol, buprenorphine, and lithium were ineffective[16-25]. These agents have a record of efficacy for other common neuropathic pain conditions, but CIPN has a different pathological origin. The American Society of Clinical Oncology (ASCO) does not recommend any agent for the prevention of CIPN[16]. A number of nonpharmacologic interventions, such as acupuncture, limb hypothermia, surgical glove (SG) compression and electrocutaneous nerve stimulation, have also been investigated. However, the paucity of randomized controlled trial evidence prohibited the inclusion of those studies in this systematic review[16].

Reducing the penetration of chemotherapeutic agents into the capillaries of hands and feet and lowering the temperature of the patient's extremities can be a way to slow blood circulation and cell metabolism in order to prevent the onset of CIPN. In fact, the tissue metabolic rate can be reduced by 50% dropping the tissue temperature by up to 10 °C. Hilotherapy is an effective method to constantly cool the temperature of the hands and feet during treatment and prevents the development of CIPN and polyneuropathy, giving patients a better QoL. In this review, we will discuss the

incidence of CIPN in gynecologic cancer patients, the clinical features related to the treatment and the way to minimize or to prevent its onset.

TAXANE-RELATED CIPN

Taxane-induced CIPN is predominantly sensory (SPN) and rarely presents with motor and autonomic changes[4]. Symptoms may arise early and even after the first administration, they are dose dependent, but tend to improve after discontinuation of treatment. However, the onset and severity are highly variable from individual to individual; no anticipatory signal can suggest a dose reduction, and there are no indications of a de-escalation of therapy to mitigate the symptoms once they arise. Furthermore, CIPN even after the end of chemotherapy can persist for months or years afterwards and in rare cases be permanent[9]. The only certain risk factor is the dose administered, with increases in risk proportional to the cumulative dose[4]. Pignata *et al*[26] found that approximately three fourths of patients treated with CP experienced peripheral neuropathy, and 7% had ≥ grade 3 CIPN. Residual neurotoxicity occurs frequently in patients after completion of CP, with a long-lasting pattern of recovery and 14% of patients still suffer residual neuropathy 1 year after treatment[27]. Such toxicity still persists in a significant proportion of patients who experience relapse and therefore, affects second-line treatment choice.

The Gynecologic Oncology Group (GOG) reported that approximately 20% of ovarian cancer, patients treated with the combination of carboplatin and PTX at 175 mg/mq every 3 wk in first-line therapy experienced grade 2 or higher neuropathy, and this percentage was increased among older patients[4]. Considering a similar total dose, the MITO-7 study showed that in patients with advanced ovarian cancer the regimen with carboplatin area under the curve (AUC) 2 mg/mL per min plus weekly PTX at 60 mg/mq was less neurotoxic than carboplatin AUC 6 mg/mL per min plus PTX at 175 mg/mq every 3 wk (grade 2 or higher neuropathy of 6% *vs* 17%, respectively). However, despite the better toxicity profile and the incremented QoL, the weekly regimen did not prolong progression free-survival (PFS)[28]. The Japanese Gynecologic Oncology Group (JGOG) randomized patients to receive carboplatin AUC 6 mg/mL per min every 3 wk plus dose dense PTX at 80 mg/mq (on days 1, 8, 15) or plus PTX at 180 mg/mq every 3 wk. The study showed that the 7% of patients treated with the dose-dense scheme had grade 3 or higher SPN and 5% had grade 3 or higher motor peripheral neuropathy (MPN), while 6% of patients treated with the standard regimen reported grade 3 or higher SPN and 4% grade 3 or higher MPN[29]. Despite a better median PFS benefit of 28.0 mo *vs* 17.2 mo and an overall survival benefit at 3 years of 72.1% *vs* 65.1% in the dose-dense treatment group compared to the conventional treatment group, more patients in the dose-dense group discontinued the treatment due to hematologic toxicity[29]. In contrast, in the JGOG-3016[30] and GOG-0262[31] studies, patients in groups 2 and 3 of ICON8[32] received up to 80 mg/mq of PTX per week and showed worse SPN in the weekly treatment group than in the every 3-wk treatment group. These findings suggest that SNP could be caused by cumulative PTX exposure rather than its dosing intensity[32]. The Scottish Ginecological Cancer Trials Group suggested that docetaxel could be less neurotoxic than PTX, and they reported a rate of 11% grade 2 or higher SPN with carboplatin AUC 5 mg/mL per min plus docetaxel 75 mg/mq *vs* a rate of 30% with carboplatin AUC 5 mg/mL plus PTX 175 mg/mq every three weeks in first-line ovarian cancer; however, this research did not demonstrate any survival benefit[33]. Unfortunately, platinum-sensitive patients who relapse are candidates for retreatment with CP, so residual peripheral neuropathy can be a limiting factor. However, studies that have evaluated the incidence of neuropathy in this setting report a 20% incidence of CIPN > 2 grade[2,34]. This proportion is lower than expected, which is a bias that is due to poor recruitment of patients with residual CIPN[2].

Symptom improvement or resolution after 3-6 mo at the end of standard treatment can be achieved by most of patient; however, in some cases, symptoms can last years, with a probability of 15% of patients still having neuropathy 6 mo after completion of chemotherapy and an 11% probability of still experiencing neuropathy at 2 years after completion of chemotherapy[4]. In the secondary analyses of the ROGY care trial among ovarian cancer patients, the course of SPN among chemotherapy-treated patients remained stable over the course of 2 years, while MPN symptoms declined at 12 mo. Furthermore, at 2 years, 13% still reported high levels of SPN[3]. Similar findings were seen in the previous PROFILE study with a prevalence of 6%-9% of SPN and 14% of MPN at 2-12 years after treatment, and a decreased in CIPN symptoms

three years after the end of treatment[6] (Table 1).

MECHANICAL SYSTEMS OF CIPN PREVENTION

Many studies have investigated the potential use of non-invasive approaches to prevent CIPN. The different mechanical systems tested include: acupuncture, scrambler therapy, hypothermia and compression therapy which aim to reduce damage to the peripheral nerves of the extremities. Acupuncture technique showed grade 3 symptom regression or stability in patients experiencing grade > 2 CIPN with weekly PTX for breast cancer[35]. Transcutaneous electrical nerve stimulation (scrambler therapy) was observed to relieve CIPN symptoms in several trials[36-41] (53% reduction in pain, 44% reduction in tingling and 37% reduction in numbness [37]), and these effects were maintained for 3 mo of follow-up[40]. The use of SG compression therapy was investigated by Tsuyuki *et al*[42] the reduction of micro-vascular flow to the hands during PTX administration allows to reduce the incidence of grade 2 or higher CIPN. The incidence of grade 2 or higher sensory CIPN, according to Common Terminology Criteria for Adverse Events (CTCAE), with SG compression therapy was low at 13.8% *vs* the incidence in the historical control group (44.1%)[42].

Cold temperature is known to reduce tissue microvascular perfusion, "therapeutic regional hypothermia" or "cryotherapy" using different limb cooling modalities (*e.g.*, direct application of ice or frozen gloves) and has been use in attempt to prevent chemotherapy-induced nail and skin toxicity, but the mechanism of the large cooling gradients of these modalities permits only intermittent coolant application and are limited by substantial intolerance and sometimes frostbite[43,44]. Rosenbaek *et al*[45] demonstrated that cryotherapy associated with PTX treatment in the adjuvant treatment of breast cancer reduces the risk of dose-limiting CIPN, and increases the number of patients receiving the full chemotherapy dose (77% of patients). However, the comparison study between cryotherapy and preventive compression therapy during treatment with nab-PTX showed no benefit for either system[46]. A recent randomized controlled trial that investigated the effect of cryotherapy administered on all four limbs during PTX treatment among early and locally advanced breast cancer patients did not decrease the incidence and severity of sensory symptoms[47]. This study was unable to conclude that cryotherapy prevents sensory neuropathy probably because of the high rates of cryotherapy interruption[47].

HILOTHERAPY A NEW PREVENTIVE APPROACH

The Hilotherm® Chemo care device is a regional cooling system that uses cuffs and piping for the hands and feet through which the coolant flows at a temperature of 10 °C. The insulators were fitted on both the hands and feet for 30 min before the start to 60 min after chemotherapy administration. A recent study in Germany evaluated the use of this device in 20 cancer patients treated with taxane-based chemotherapy as a preventative method. No patients developed grade 2 or 3 symptoms of CIPN. Four weeks after their chemotherapy ended, 70% of patients who used hilotherapy had no symptoms at all. The other 30% of patients were found to have grade 1 symptoms. Although cooling from the start of treatment achieved the best outcome, the group of patients (n = 36) who started hilotherapy when CIPN symptoms occurred showed a reduction in severe symptoms of CIPN. The use of hilotherapy was found to be comfortable and safe[48].

OUR PERSONAL EXPERIENCE

In the published small retrospective trial from our center we enrolled 64 patients with breast, gynecologic and pancreatic cancer to wear Hilotherm cuffs and piping on their hands and feet. Fifty-four (84%) of these patients completed all cooling cycles during their taxane-based chemotherapy. Continuous cooling was well tolerated by all patients. No patients had grade > 2 CIPN or had serious or lasting side effects as a result of hilotherapy. The median time to CIPN onset was 77 d for the entire population[49].

The updated data in the subgroup of patients with gynecological cancer treated with the combination of carboplatin AUC 5 mg/mL per min plus PTX at 175 mg/mq

Table 1 Incidence of chemotherapy-induced peripheral neuropathy

Trail	Indication	No. of patients	Treatment	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)
AGO OVAR 3[35]	FIGO IIB-IV fist line	388	CP	39	28	6.7
		384	PP	33	36	12.5
JGOG[29]	FIGO II-IV fist line	319	CP			20 (6)
		312	C-P			21 (7)
MITO 4[27]	FIGO IC-IV fist line	120	CP	51 (42)	13 (11)	1 (1)
MITO 2[26]	FIGO IC-IV fist line	407	CP	115 (28)	64 (16)	12 (3)
		396	C-PLD	47 (12)	11 (3)	1 (< 1)
MITO 7[28]	FIGO IC-IV fist line	404	CP	95 (24)	58 (15)	8 (2)
		406	CP weekly	98 (25)	24 (6)	0
ICON 4[2]	Platinum-sensitive relapse > 6 mo	410	P-based		4 (1)	
		392	CP/PP		76 (20)	

CP: Carboplatin area under the curve (AUC) 5/6 mg/mL per min plus paclitaxel 175-185 mg/mq every 3 wk; CP weekly: Carboplatin AUC 2 mg/mL per min plus paclitaxel 60 mg/mq every week; C-P: Carboplatin AUC 6 mg/mL per min every 3 wk plus paclitaxel 80 mg/mq on days 1, 8, 15, and every 21 d; C-PLD: Carboplatin AUC 5 mg/mL per minute plus pegylated liposomial doxorubicin 30 mg/mq every 3 wk; P-based: Cisplatin (50 mg/mq in combination with other drugs or 75 mg/mq as a single agent)/carboplatin AUC 5/6 mg/mL every 3 wk; PP: Cisplatin 75 mg/mq plus paclitaxel 175 mg/mq every 3 wk.

every 3 wk show that 35 of 44 patients (79.5%) completed all the planned cycles of chemotherapy with the cooling system. The severity of PN was evaluated by a nurse using the CTCAE (2017). Statistical analyses were performed using STATA 13.0 (StataCorp LP, Collage Station, TX, United States). Only 1 patient (2.2%) had grade ≥ 2 CIPN after 4 cycles of CP and completed the last cycle with carboplatin monotherapy; 2 patients (4.5%) stopped hilotherapy due to excessive coldness; 3 patients (6.8%) stopped treatment due to disease progression and 2 patients (4.5%) stopped the treatment due to PTX allergic reaction; 3 patients (6.8%) had PTX reduction/interruption that was due to PTX hematologic toxicity and completed the cycles with carboplatin monotherapy and 3 patients (6.8%) concluded the treatment after 3/4 cycles due to hospitalization for planned surgery. Follow-up data are premature at the time of writing this review. Tables 2-4 show patient characteristics and results.

DISCUSSION

Neuropathy is an important, dose-limiting, painful, and often irreversible toxicity associated with taxane therapy. Approximately 20% of women treated for ovarian cancer show CIPN, and this toxicity impairs their QoL and can persist for months or even years after the end of treatment. Instead, for recurrent disease the established treatment is a reintroduction of the first-line scheme in sensitive patients, with a with a higher probability of developing toxicity or worsening of pre-existing toxicity with a consequent limitation of the duration of treatment. The only prophylactic therapy for CIPN suggested by the guidelines of the ASCO 2014[16] is duloxetine, but it has limited efficacy in chemotherapy-induced neuropathic pain and has no benefit on paraesthesia and functional disability. Recently, ganglioside monosialic acid has shown promising effects on the prevention of neuropathic symptoms with a lower incidence of grade> 1 on the CTCAE v4.0 scale (14.3% *vs* 100.0%, $P < 0.001$) both sensory (26.4% *vs* 97.8%, $P < 0.001$) and motor (20.9% *vs* 81.5%, $P < 0.001$) in patients with breast cancer treated with PTX[50]. Previous studies suggested positive effects of physical activity on CIPN with a higher CIPN in patients who do not meet recommended activity levels[51,52]. Moderate to vigorous activity was associated with lower CIPN in taxane-treated patients[53]. Patients who followed physical activity guidelines reported a longer time to the onset of CIPN since completing chemotherapy, potentially suggesting adaptation to neuropathic symptoms over time, enabling the return to physical activity[53]. Conveying PTX using lipid-based nanoformulations represents a potential strategy to overcome PTX side effects. In a recent

Table 2 Patient characteristics

Characteristics	*n* (%)
Age, mean (range)	61 (35-74)
Gyneacologic cancer	44
Risk factors	
None	38 (86.3)
Diabetes	3 (6.8)
Arthrosis and diabetes	1 (2.3)
Hyphotyroidsm	2 (4.6)

Table 3 Chemotherapy-induced peripheral neuropathy per cycle, according to Common Terminology Criteria for Adverse Events (version 4.0)

Chemotherapy	Number of patients	Reported CIPN, *n* (%)		
		Grade 0	Grade 1	Grade ≥ 2
Cycle 1	44	44 (100)	0 (0)	0 (0)
Cycle 2	44	43 (97.7)	0 (0)	0 (0)
Cycle 3	43	40 (93.0)	1 (2.3)	0 (0)
Cycle 4	41	33 (80.5)	1 (2.4)	0 (0)
Cycle 5	34	22 (64.7)	1 (2.9)	1 (2.9)
Cycle 6	24	20 (83.3)	2 (8.3)	1 (4.2)

CIPN: Chemotherapy-induced peripheral neuropathy.

Table 4 Chemotherapy (paclitaxel) dose changes during hilotherapy treatment

	Patients, *n* (%)
Completed scheduled CP cycle[1]	37 (84.1)
Paclitaxel dose reduction/discontinuation	9 (20)
Reason for Paclitaxel reduction/discontinuation	3 (6.8) hematological toxicity; 3 (6.8) disease progression; 2 (4.5) paclitaxel allergic reaction; 1 (2.2) CIPN ≥ 2

[1]Carboplatin area under the curve 5 mg/mL per min plus paclitaxel at 175 mg/mq every 3 wk.
CP: Carboplatin/paclitaxel; CIPN: Chemotherapy-induced peripheral neuropathy.

study, this formulation was tested on lung tumor cells and *in vivo* and showed a very low concentration in the dorsal root ganglia, where PTX accumulates and causes damage to the somas of peripheral sensory neurons[54]. This approach could lead to a reduction in the incidence of peripheral neuropathy and be the subject of study in gynecological cancers.

Different mechanical system of CIPN prevention were tested and founded that vasoconstriction and cold reduce drug exposure during chemotherapy infusion[55], but the limits of cooling systems are primarily the cold intolerance and cold-related injuries. The small single-arm trial from our center that investigated the effects of hilotherm cooling system demonstrate to prevent CIPN[49]. The advantages of continuous cooling are a more tolerable and controlled temperature reduction for the duration of chemotherapy instead of ice or frozen gloves. Continuous cooling whit a coolant temperature of 10 °C continued for the duration of chemotherapy and was well tolerated. In the ovarian cancer subgroup only one patient showed CIPN ≥ 2, and only two patients (4.5%) stopped halotherapy for cold intolerance. Published data showed that no patients reported the appearance of CIPN symptom at a follow-up time of 6 mo[49]. We expect to maintain these follow-up results also from the new

enrolled patients.

CONCLUSION

The prevalence of CIPN was approximately 68% within the first month following the end of taxane chemotherapy treatment and 30% after 6 mo or more[4]. In a survey of ovarian cancer patients, CIPN symptoms were associated with lower functioning and greater health-related worry[5]. Additionally, a survey covering various cancer types found that patients reporting neurotoxicity had higher symptom burden and stress, and poorer QoL[56]. Reducing the incidence of neuropathy to improve the patient's QoL and avoiding discontinuation or dose reduction of anticancer treatment is of primary importance. Unfortunately, comprehensive knowledge of the mechanisms leading to CIPN is still lacking and no drug has been approved to prevent or cure it. Hilotherapy has proven to be a safe, well tolerated and effective method of preventing and treating CIPN. To validate the use of hilotherapy as a preventive method a large-scale trial and a longer follow up are necessary.

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ORIGINAL ARTICLE

Clinical and Translational Research

Impact of community-based exercise program participation on aerobic capacity in women with and without breast cancer

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Abstract

BACKGROUND

Evidence for exercise as an efficacious strategy to improve aerobic capacity of breast cancer survivors (BCS) has come largely from intervention studies conducted in laboratory settings. There is an increasing need to translate to community-type settings, but the efficacy of those interventions using gold standard evaluation is not well-established.

AIM

To investigate whether similar improvement in aerobic capacity (maximal oxygen consumption [VO_2]) measured with gold standard testing can be achieved through a community-based setting in BCS.

METHODS

A peak cardiopulmonary exercise test (VO_{2peak}), 6-min walk test (6MWT), and timed up and go test (TUG) were assessed pre- and post-16 wk of progressive intensity aerobic and strength training exercise at a community center.

RESULTS

The sample consisted of 31 early BCS (< 1 year since treatment completion) and 15

controls (CTLs). Both groups significantly improved VO_{2peak} (+1.2 mL/kg/min; P = 0.030), 6MWT (+35 meters; $P < 0.001$), and TUG (-0.44 s; $P < 0.01$) following training. Both groups improved peak cycling power during the cardiopulmonary exercise test with BCS improving by +10 watts more than the CTLs (P = 0.020). Average exercise attendance was 71% (34 of 48 possible days), but compliant days averaged only 60% of total days for aerobic, and < 40% for strength in both groups.

CONCLUSION

Community-based exercise programs can be an effective strategy to improve aerobic capacity and physical function for early-stage BCS but potentially not to the same extent observed in laboratory-based randomized controlled trials. Further research is needed to explore barriers and facilitators of exercise engagement in community-based centers to maximize training benefits for adults with cancer.

Key Words: Aerobic capacity; Breast cancer; Community-based; Exercise; Physical function

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Core Tip: Breast cancer survivors can improve aerobic capacity and physical function through participation in community-based exercise programs. However, these improvements may not be as substantial as those observed in laboratory-based randomized controlled trials. While community-based programs may provide cancer survivors better flexibility and access than laboratory settings, to maximize training benefits, continued work developing and testing exercise intervention prescriptions and associated outcomes is necessary in addition to exploring barriers and facilitators of exercise engagement for adults with cancer.

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INTRODUCTION

Cardiorespiratory fitness describes the ability to use oxygen to produce energy for physical work and can be quantified as aerobic capacity (maximal oxygen consumption [VO_2]; mL O_2/kg/min) using the gold standard maximal cardiopulmonary exercise testing (CPET). Breast cancer survivors (BCS) have substantially impaired aerobic capacities[1,2], with a decline in VO_2 similar to that experienced with a 10 year increase in age, which can be attributed to both cancer treatment-related toxicities and increased sedentary lifestyle habits[3,4]. This impairment places BCS at substantial risk of death from cardiovascular (CV) disease and loss of functional independence[5]. Fortunately, exercise is a known promoter of CV health in the general population[6] and is an effective strategy for improving aerobic capacity in BCS during and post-treatment[7-9].

Multiple well-designed and controlled exercise intervention trials have demonstrated the benefits of aerobic and strength training exercise on aerobic capacity and other physical function outcomes, resulting in the development of exercise prescription guidelines for adults with cancer[10]. Guidelines were recently updated in 2019 and recommend at least 30 min of moderate intensity aerobic exercise at least 3 d/wk, and at least 2 d/wk of strength training for people with cancer[11]. Participation in exercise interventions reflecting these guidelines have demonstrated increases in aerobic capacity by 2.3-2.9 mL/kg/min (approximately 10%-15%)[7,9,12]. However, these changes in aerobic capacity have been observed primarily from

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interventions in highly controlled laboratory randomized controlled trials (RCTs), and the efficacy of this "trial-proven" exercise prescription delivered through real-world settings such as community-based centers has not been well-studied[13]. These settings likely differ from laboratory settings because they may be more relaxed environments with the opportunity for increased socialization between participants, and may provide additional flexibility in terms of training modes and session attendance (ATT) than what laboratory-controlled trials may provide or allow, regardless that the intention to improve fitness and quality of life may be the same. Of the limited work available on evaluating outcomes in community-based settings, cardiorespiratory fitness appears to be maintained or improved but has not been evaluated using direct, gold standard exercise testing methods (*i.e.* maximal CPET)[14-17]. Instead, alternative indicators of functional capacity such as the 6-min walk test (6MWT), and the timed up and go (TUG) test have been used, which may offer improved utility and feasibility in clinical/community-based centers. As exercise is increasingly recommended to survivors, and as interventions transition from highly controlled laboratory based settings to more practical venues like community-based centers, it is important to precisely evaluate whether similar cardiorespiratory fitness benefits can still be achieved to properly support and accommodate a large volume of cancer survivors in need of improving or even maintaining their overall health, physical function, and quality of life through participation in regular exercise[13,18].

Exercise programming for cancer survivors is a well-recognized challenge in exercise oncology[19-21] because survivors frequently require tailored exercise accommodations based on cancer-related treatments and associated side effects. Oncology-trained staff in community-based settings may be uniquely positioned to modify workouts, but these necessary adjustments have the potential to significantly impact exercise engagement[20,22]. Therefore, collecting and reporting exercise efficacy, ATT, and compliance in community-based intervention studies will improve understanding of how patients engage in these settings and how exercise engagement may relate to physical fitness and health outcomes[23-25].

The primary aim of this study was to evaluate the impact of a community-based exercise program on change in aerobic capacity in women with breast cancer measured using the gold standard CPET. A secondary aim was to evaluate if changes in aerobic capacity in the community-based setting differ between women with and without cancer. A third aim evaluated how exercise engagement (ATT and compliance) relates to changes in aerobic capacity. For the primary aim, we hypothesized that aerobic capacity would significantly improve following community-based training. For the second and third aims, we hypothesized that women in both groups would benefit similarly from the community-based protocol and that the observed changes in aerobic capacity would be directly related to training ATT and compliance.

MATERIALS AND METHODS

This study used a parallel-group, pre-post design. Two groups (one BCS, one non-cancer control [CTL]) of women were engaged in an identical supervised exercise evaluation and intervention at Get REAL and Heel (GRH), an established, community-based exercise facility for cancer survivors in Chapel Hill, North Carolina that has offered exercise training reflective of recommended guidelines to cancer survivors for the past 10 years. This study leveraged an associated exercise oncology lab with gold standard testing capacity to evaluate the existing community-based program as it has currently operated. For data collection, two consecutive visits to the Exercise Oncology Research Laboratory of the UNC Department of Exercise and Sport Science occurred prior to (pre-intervention) and immediately following (post-intervention) the 16-wk exercise program. Outcomes evaluated included peak aerobic capacity (VO_{2peak}), the 6MWT, and the TUG test. Patient demographics and cancer-specific clinical data were extracted from the UNC Health Care electronic medical record by the research team. The study (NCT03760536) was approved by the Protocol Review Committee of the UNC Lineberger Comprehensive Cancer Center and the UNC Institutional Review Board. All participants provided written informed consent.

Study participants

Women ≥ 21-years-old diagnosed with early-stage (Stage 0-III) breast cancer and within 1 year of completing primary therapy (surgery, chemotherapy, radiation) were eligible for the BCS arm. Women in the CTL group were age-matched with no history of cancer. Both groups self-reported no more than 2 d of physical activity per week

and not meeting recommended physical activity guidelines. Both groups self-reported no cardiovascular, metabolic, or orthopedic limitations, and were cleared by a cardiologist (both groups) and oncologists (BCS only) prior to participating. Participants in the BCS group were recruited from the Medical Oncology clinic at the North Carolina Cancer Hospital, and by word of mouth from local oncologists and cancer centers. Participants in the CTL group were residents from nearby communities and recruited *via* electronic and paper fliers and word of mouth. The recruitment period was April 2017-2019 for BCS and February to August 2019 for CTL.

Exercise intervention

Both BCS and CTL groups participated in a 16-wk supervised, small-group (approximately 4-8 participants per session) exercise intervention including combination aerobic and strength exercise training 3 d a week for approximately 1-h total per d (48 total days of training opportunity) at the GRH facility. A variety of equipment for training was used and adapted to individual participant fitness and mobility needs, allowing trainers to maximize patient safety and exercise engagement while helping participants strive towards reaching guideline-prescribed levels of activity. Participants could choose treadmills, stationary bikes or ellipticals for aerobic work and body weight, resistance bands, dumbbells, or machine weights for strength training, depending on their abilities and preferences.

The specific exercise prescription for the supervised intervention at the GRH facility is presented in Table 1. In the first 2 wk, all study participants were asked to engage in low intensity aerobic exercise for 10-15 min plus approximately 30 min of light-to-moderate strength training each day at the facility. Participants were encouraged to challenge themselves to safely engage in exercise intensity that elicited the prescribed exertion level (Borg's Rating of Perceived Exertion[26], RPE) throughout the intervention. RPE was used as a surrogate measure of intensity in this study in place of heart rate monitoring since it has historically been used at GRH because heart rate transmitter signals tended to overlap between participants in the limited training space available. Intensity could be increased under the direction of trained exercise staff by adding grade (treadmill), resistance (cycling), or speed (mph or rpm) for aerobic training, and by progressing from body weight to resistance bands to free weights or machines for resistance training. The exercise training program has been implemented and designed to create a dose-response to help participants safely attain weekly exercise goals reflective of national guidelines[11].

Data collection

Aerobic capacity: Participants completed a familiarization session for the maximal CPET on the first day of data collection using a Corival (Lode B.V., Groningen, The Netherlands) electronically-braked cycle ergometer. Participants were fitted with a Polar heart rate monitor (Polar Electro Inc., Lake Success, NY, United States) and a mask for indirect calorimetry using the ParvoMedics metabolic system. No gas was collected or analyzed on the first day but participants completed a 5-min unloaded and 20 watts loaded cycling warm-up followed by an incremental 15 watts/min with a protocol up to 75% heart rate reserve for familiarization. On day 2 of data collection, participants completed the same CPET protocol but continued until they reached volitional exhaustion or were stopped when oxygen consumption plateaued despite an increase in wattage. Testing was also terminated if cadence was < 50 rpm. Gas exchange data were exported in 5 s average bins, and VO_{2peak} was recorded as the average of the three highest recordings within the final minute of the maximal test. Time to test termination (TTE) was recorded as the ramp-only portion (excluding the 5-min warm up portion), and peak power was the highest wattage recorded before test termination and were used as additional descriptive data for better interpretation of testing performance. This protocol was repeated at the end of the 16-wk exercise intervention.

Intervention ATT and compliance: Training records were maintained by GRH staff to track participant ATT and compliance over 16 wk. ATT was calculated as the number of days participants came to the facility out of 48 total days of opportunity. Compliance was also included in our study to: help distinguish ATT from fulfillment of the workout prescription; and to quantify completed exercise load and progression, which improves the ability to evaluate effects of the intervention on VO_{2peak} with more granularity than ATT alone. An aerobic compliant (aCOMP) day was defined as the participant achieving ≥ 80% of the prescribed duration of aerobic exercise within the prescribed RPE range (Borg 6-20 scale[26]). A strength compliant (sCOMP) day was defined as the participant achieving ≥ 80% of prescribed strength volume (sets x

Table 1 Exercise progression in the Get REAL & HEEL Exercise Program

Aerobic exercise	Week 1-2	Week 3-7	Week 8-16
Duration (min)	10-15	10-30	30
Intensity	Low		Moderate
RPE	8-11		12-14
Resistance exercise	**Week 1-2**	**Week 3-7**	**Week 8-16**
Duration (min)	~30		
Intensity	Low to moderate		High
RPE	7-13		14-15
Sets x Reps / exercise	1 x 15	2 x 10 - 15	2 x 10

repetitions) within the prescribed RPE range. The 80% duration/volume cutoff in conjunction with RPE as an indicator of intensity was used to help quantify specific training load, not just ATT, and was determined by an experienced exercise oncology research team member as training criteria reasonable to induce changes in aerobic capacity.

Additional outcomes: 6MWT and TUG. On the first day of data collection and prior to CPET familiarization, participants completed one 6MWT and two TUG tests to assess functional capacity. The fastest TUG time was recorded for analysis. This protocol was repeated at the end of the 16-wk intervention. These outcomes were included for additional context about participant performance and may be useful for reference/implementation in future work as a more clinically feasible measure of cardiorespiratory fitness and overall functionality alternate to gold standard VO_{2peak} outcomes, since most community-based program usually do not have the capacity to conduct maximal CPETs.

Power calculations

Published systematic reviews and meta analyses support exercise therapy to increase aerobic capacity in breast cancer survivors by approximately 2.3-2.9 mL O_2/kg/min[7,9,12] in laboratory RCTs. For our analysis, power was calculated based on a primary outcome of mean difference in delta VO_{2peak} of BCS following training. Given 80% power and an alpha of 0.05, and assuming a mean difference following training of 2.5 mL O_2/kg/min and standard deviation of 3 (d = 0.83), 11 BCS participants would be required. Oversampling was performed to account for potential dropouts and missing data.

Statistical analyses

Statistical analyses were performed using Jamovi open source software (The Jamovi project, version 1.2.5). Baseline descriptive statistics were computed to summarize participant demographics, breast cancer diagnosis/treatment characteristics, and ATT and compliance with the exercise intervention. The α-level was set *a priori* for all statistical procedures at < 0.05.

Aerobic capacity following training: The primary aim of this study was to evaluate the impact of a community-based exercise program on change in aerobic capacity (deltaVO$_{2peak}$) in women with breast cancer measured using the gold standard CPET.

Aerobic capacity between groups following training: A linear mixed model was used to evaluate the impact of the exercise training program on change in aerobic capacity (deltaVO$_{2peak}$) between women with and without cancer. The model used fixed effects of time (pre- *vs* post-testing) and condition (BCS *vs* CTL), and a random effect of subject with adjustment for age. If time-by-condition interactions were not significant, the final models estimated the main effects of condition and time on each outcome. Cohen's d effect size was calculated per group as the mean difference from pre- to post-testing, divided by the standard deviation (SD) of the mean difference. For *a priori* interpretation of effect sizes, Cohen's "rules of thumb" were used: small = 0.20, medium = 0.50, and large = 0.80[27].

Intervention ATT and compliance: Independent samples *t*-tests were used to compare exercise ATT and compliance between groups after the intervention. Univariate linear regression models were used to evaluate associations of deltaVO_{2peak} with days of exercise ATT, days of aCOMP, and days of sCOMP per group. For an exploratory analysis, the two groups were also pooled and reevaluated.

TTE, peak power, 6MWT, and TUG: Separate linear mixed models were used to evaluate the effects of time (pre- *vs* post-testing) and condition (BCS *vs* CTL) on time to test termination, peak power, 6MW, and TUG. If time-by-condition interactions were not significant, the final models estimated the main effects of condition and time on each outcome. These analyses were completed to provide supplemental information about the performance changes from participating in a real-world community-based program; programs that provided BCS with flexible opportunities to engage in regular supervised exercise training.

RESULTS

Thirty-five women with breast cancer were enrolled, of which 31 completed all study assessments. Twenty-one non-cancer CTLs were enrolled, of which 15 completed all study assessments (Figure 1). Those who did not complete post-testing were excluded from the analyses due to missing data but were otherwise very similar to women with complete data. At baseline (Table 2), BCS participants were taller ($P = 0.035$) and had worse TUG performance ($P < 0.01$) than CTL participants.

Aerobic capacity between settings

Women with breast cancer improved VO_{2peak} following community-based exercise training by 1.2 (3.3) mL O_2/kg/min.

Aerobic capacity between participants

There was no significant time x group interaction for VO_{2peak} but women in both the BCS and CTL groups had significantly improved aerobic capacity by approximately 1.2 mL O_2/kg/min (95%confidence interval [CI]: 0.15-2.27, Cohen's $d = 0.36$; $P = 0.03$) from pre- to post-testing (Figure 2A).

Intervention ATT and compliance

Both groups attended the same mean number of training sessions (approximately 34 d of 48 planned; $P = 0.420$) for an average ATT of 71% (Table 3). Differences in days of aerobic compliance between groups did not reach statistical significance but demonstrated a medium to large effect size (Cohen's $d = 0.63$; $P = 0.06$) with CTL exhibiting more days of aerobic compliance than BCS - 32 (9) *vs* 26 (10) d. Control participants also completed a greater number of compliant strength training days compared to BCS - 18 (3) *vs* 14 (5) d (Cohen's $d = 1.0$; $P = 0.011$). The total number of compliant strength training days was < 40% total days in both groups. There were no significant associations found in univariate analyses between deltaVO_{2peak} and ATT or compliance for either group, independently or in the pooled sample (data not shown).

TTE, peak power, 6MWT, and TUG

There was a significant time x group interaction for peak power with both groups demonstrating increased power at post-testing similar to other studies[23], but BCS increased by approximately 10 more watts than the CTLs (95%CI: 1.8-17.5; $P = 0.02$) (Figure 2B). A significant main effect of time was observed for TTE and 6MWT. Both groups completed almost a minute more of exercise during the peak test (95%CI: 0.60-1.29; $P < 0.001$) (Figure 2C) and walked approximately 35 meters more in 6 min (95%CI: 21.5-49.1; $P < 0.001$), which is considered clinically meaningful in clinical populations[28,29], at post-testing. Significant main effects of time and group were observed for TUG with both groups improving time by approximately 0.4 s (95%CI: -0.72-(-0.17); $P < 0.01$) with an approximately 0.8 s difference between groups at baseline sustained after exercise training (95%CI: 0.21-1.5; $P = 0.012$).

Table 2 Baseline characteristics – mean (SD)

	Breast cancer (*n* = 31)	Control (*n* = 15)	Total (*n* = 46)
Demographics			
Age (yr)	54 (12)	55 (8)	54 (11)
Height (cm)	167 (7)	162 (7)[1]	165 (8)
Weight (kg)	77 (12)	75 (14)	76 (13)
BMI categories (kg/m^2)			
Normal (18.5 to < 25)	29%	13%	24%
Overweight (25 to < 30)	48%	60%	52%
Obese (30 to < 35)	10%	7%	9%
Obese II (≥ 35)	13%	20%	15%
Body fat (%)	41 (6)	40 (4)	41 (5)
Lean mass (kg)	42 (6)	42 (7)	42 (6)
Postmenopausal (%)	65%	67%	65%
Race (Caucasian, %)	87%	100%	91%
Clinical variables			
VO_{2peak} (mL/kg/min)	20.9 (5.3)	22.4 (2.8)	21.4 (4.6)
Time toexertion (mm:ss)	9:41 (1:42)	10:21 (1:20)	9:54 (1:36)
Peak power (Watt)	120 (26)	130 (20)	123 (24)
Six minute walk (m)	538 (72)	557 (53)	544 (67)
Timed up & go (sec)	4.8 (1.2)	3.9 (0.7)[1]	4.5 (1.2)
Breast cancer details			
Tumor stage			
0	3%	--	--
I	27%	--	--
II	47%	--	--
III	23%	--	--
HR status			
ER positive	81%	--	--
HER2 status			
Positive (all received trastuzumab)	26%	--	--
Surgery			
Lumpectomy	71%	--	--
Mastectomy	29%	--	--
Cardiotoxic therapies			
Anthracycline	23%	--	--
Trastuzumab	26%	--	--
Anthra + Tras	3%	--	--
Endocrine therapy			
Aromatase inhibitor	45%	--	--
Tamoxifen	19%	--	--
Days since end of primary treatment	101 (91)	--	--

[1]*P* value < 0.05, significantly different between groups.

Table 3 Attendance and compliance (out of 48 total days of training opportunity)

	Breast cancer mean (SD) (*n* = 31)	Control mean (SD) (*n* = 15)	Cohen's d	*P* value
Intervention Attendance (days)	35 (9)	33 (9)	0.22	0.420
Aerobic Compliance (days)	26 (10)	32 (9)	-0.63	0.060
Strength Compliance (days)	14 (5)	18 (3)	-1.00	0.011

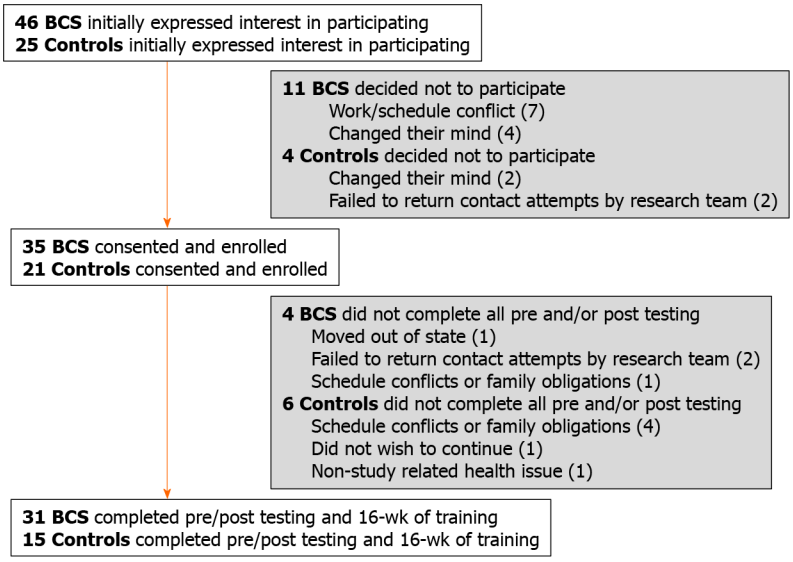


Figure 1 Recruitment and retention.

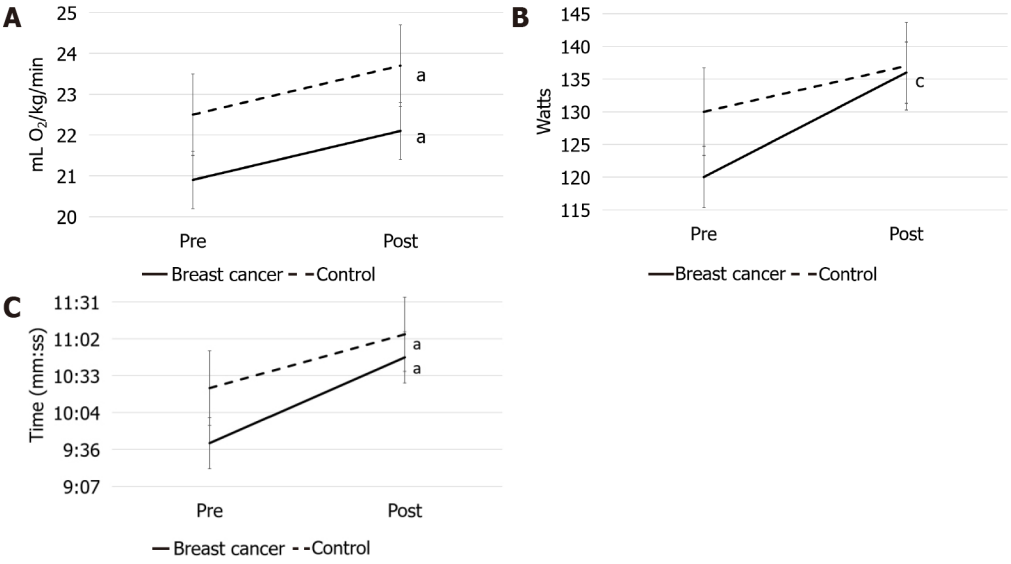


Figure 2 Maximal testing results (standard error bars). A: Aerobic capacity; B: Peak Power; C: Time to Exhaustion in breast cancer survivors and controls before and after 16-wk of training. [a]*P* < 0.05 for time; [c]*P* < 0.05 for group time interaction.

DISCUSSION

Participation in community-based exercise training reflective of recommended guidelines increased aerobic capacity in women with breast cancer in our study, but not to the extent observed in laboratory RCT settings. The 1.2 mL O_2/kg/min (+6%) improvement following community-based training is less than the 2.3-2.9 mL/kg/min (approximately 10%-15%) improvement reported in recent meta-analyses; however, the latter interventions were completed in laboratory RCT settings[7,9,12]. Changes in VO_{2peak} following training in non-RCT settings have been only minimally evaluated using gold standard methodology[13] in contrast to alternatives such as submaximal VO_{2peak} testing, 6MWT, and/or TUG[14,16,17,30] but have nonetheless supported beneficial changes. However, while the transition of interventions from laboratory settings to community settings is increasing[13,31,32], it is important to evaluate outcomes with gold standard testing to ensure survivors are receiving effective intervention in this newer setting. While the aerobic capacity improvement in our study may be small, substantial decline in aerobic capacity is a known manifestation of cancer survivorship[1,3,33]. Therefore, maintenance or even slight improvement is encouraging especially in conjunction with the observed improvements in peak cycling power and cycling endurance. Furthermore, while functional capacity (6MWT and TUG) of BCS in our study is not equivalent to non-cancer population norms[34], it reflects improvement commonly observed for women with breast cancer[35], which is of clinical relevance[28,29] and underscores the beneficial impact of exercise for demands of daily life.

Both women with and without breast cancer in our study demonstrated similar improvements in aerobic capacity (+1.2 mL/kg/min; $P = 0.03$) following the community-based exercise program. Interestingly, VO_{2peak} of both groups reflected that of published norms for women with breast cancer who are post-treatment (21.5 mL O_2/kg/min)[2]. For CTL participants, this is substantially lower than conventional standards[36]. Recently, our laboratory has consistently observed impaired VO_{2peak} in both BCS and middle-aged, sedentary women without cancer[37,38]. We speculate that this may reflect a regional fitness characteristic but warrants further investigation. In terms of training response for non-cancer populations, interventions similar to our study have generally elicited up to a 15% improvement in VO_{2peak}[39,40]. Therefore, the approximate 6% improvement observed in our study is also less than what is considered clinically significant in non-cancer populations[41,42]. The relatively poor compliance observed in our study indicates that prescribed training progression was not followed and may provide a partial explanation for the relatively modest improvements observed in $VO2_{peak}$. Exercise dosing and progression are important components related to the FITT principle[20,21,43] and are important factors for impacting patient physical and functional outcomes[8,11,44]. Furthermore, while ATT in our study (71%, attending about 2 of 3 training days per week) is similar to other exercise oncology interventions (approximately 70%-75%)[19,20,45], it reflects less than that recommended by national guidelines (≥ 3 d/wk, ≥ 30 min/d)[11]. It is reasonable to consider that improved exercise ATT closer to or matching the established, recommended guidelines in addition to improved compliance (which considers progressing intensity) would lead to more optimal physiological changes. However, despite modest intervention engagement, participants in our study still demonstrated positive/beneficial training adaptations which reiterates the need to investigate the fundamental question of what is the optimal training prescription for this population [9,23,43,46].

The ability to provide a community-based program that may improve physical and functional health and that survivors enjoy is paramount, especially when a survivor needs to prioritize aspects of work, life, and family around exercise[20,46]. Documenting exactly why patients missed sessions was not specifically recorded in this study but were anecdotally related to job, family, or general life obligations. Survivors also frequently expressed enjoyment with study participation and the GRH program, and many women regretted not having started their exercise journey earlier in treatment process. With respect to our generally positive findings, community-based programs such as GRH appear to have a place in cancer survivorship. Continued focus and conversations with survivors to better understand challenges around exercise compliance may help identify targets to improve engagement, and should remain a priority.

Difficulties with exercise engagement and compliance reflect the well-recognized challenges of exercise programming for clinical populations[19-21,47,48]. Based on the observations of our exercise staff and research team, participant compliance was primarily hindered by not achieving prescribed intensity, as measured by self-reported

RPE. This method has the inherent weakness of subjectivity; however, it was the most feasible option in our relatively small community-based setting where, historically, heart rate monitor transmitter signals have overlapped between participants, displayed incorrectly, and caused unnecessary worry/confusion to an otherwise upbeat and positive environment. In our study, it was observed that while participants would and could complete both prescribed duration (aerobic) and volume (sets x reps, strength), there was reluctance to increase intensity (grade/speed/weight, *etc.*) especially in the second half of training when intensity targets increased. Strength training intensity targets were achieved less frequently than aerobic for both groups, leading to fewer strength compliant days than aerobic (Table 3), which has been observed previously in this population[20,49,50]. GRH training staff encouraged and guided participants how to safely reach more difficult intensities and no adverse events were documented during training; therefore, lack of training support, injury, or unnecessary discomfort were unlikely contributors to sub-optimal compliance, yet successfully increasing intensity remained a challenge. While participants were reminded how to contextualize and use the RPE scale, it is possible there was misunderstanding or concern from participants that reporting a higher RPE somehow suggested they were less capable of completing the prescribed exercise.

The primary limitation of our study is that it was a small, self-selected sample of mostly White women willing who were able to engage in exercise training for 4 consecutive months. This is not representative of the majority of women with breast cancer, especially younger women who may have acute family and job demands. The greatest strength of this study was the well-established, long-standing community-based exercise oncology program with veteran training staff, but we recognize that this experienced environment is difficult to replicate[13,51-53] especially in partnership with facilities capable of gold standard testing.

Future research would benefit from the inclusion of more diverse participants and from continued efforts to explore barriers and facilitators of exercise engagement. Calculation of both ATT and compliance in community-based settings similar to the methods presented in our study would improve the ability to quantify exercise load, progression, and dose responses more precisely. Heart rate monitoring in addition to self-reported RPE may also help better communicate and indicate training intensity, especially as technology for self-monitoring progresses. Directly collecting more information from participants about missed sessions and live feedback during training sessions would help provide better understanding of participant barriers, concerns, and potential limitations to engagement. These improvements would help contribute to the development of more specific future exercise prescriptions that are both suitable and effective in community-based settings. Furthermore, a third group of BCS who did not exercise for 16-wk, or who completed prescriptions with differing levels of exercise volume (days/reps/time) and/or undulating intensity progression (high *vs* low intensity) would help clarify specific findings from the current study and enhance future designs.

CONCLUSION

A community-based exercise program such as GRH can improve aerobic capacity and overall physical function in women with breast cancer, potentially not to the extent of an exercise oncology laboratory based RCT setting but similar to that of women without a cancer diagnosis participating in a similar program. Survivors may not meet recommended physical activity guidelines immediately, but proper training accommodations may help facilitate and encourage the integration of exercise as a daily routine. This is an important first step to life-long exercise commitment and provides the foundation to achieve recommended guidelines. Community-based venues offering exercise oncology programs have the potential to augment long term cancer care[13], will be essential for accommodating the growing number and needs of survivors[18], and should be continually evaluated as interventions are implemented in new settings.

ARTICLE HIGHLIGHTS

Research background

Exercise is an efficacious strategy to improve aerobic capacity of breast cancer

survivors (BCS) but has not been consistently evaluated with gold standard testing in community-based settings.

Research motivation

As a growing number of BCS are in need of community-based exercise access, providing effective interventions is paramount to their long term health and functionality.

Research objectives

The objective was to use gold standard testing to determine whether breast cancer survivors exhibit similar improvement in aerobic capacity (maximal oxygen consumption [VO_2]) following community-based exercise compared to interventions in laboratory settings.

Research methods

A peak cardiopulmonary exercise test (VO_{2peak}), 6-min walk test (6MWT), and timed up and go test (TUG) were assessed pre- and post-16 wk of progressive intensity aerobic and strength training exercise at a community center.

Research results

Both BCS (n = 31) and CTL (n = 15) groups significantly improved physical and functional capacity following training (VO_{2peak} +1.2 mL/kg/min, P = 0.030; 6MWT +35 meters, P < 0.001; TUG -0.44 s; P < 0.01). Peak cycling power improved in both groups with BCS exhibiting 10 watts more improvement than CTL (P = 0.020). Average exercise ATT of 71%, 34/48 possible days) is in accordance with previous work, and the modest compliance (60% aerobic, < 40% resistance) emphasizes the challenges of exercise engagement in clinical populations.

Research conclusions

A community-based exercise program can effectively improve aerobic capacity and physical function for early stage breast cancer survivors but potentially not to the same extent observed in laboratory-based randomized controlled trials. Further research is needed to explore barriers and facilitators of exercise engagement in community-based centers to maximize training benefits for adults with cancer.

Research perspectives

Providing BCS with accessible and effective exercise interventions is a critical component in survivorship and should be continually evaluated with gold standard outcomes, especially because it is not yet a standard intervention of oncology practice.

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META-ANALYSIS

Phytochemically rich dietary components and the risk of colorectal cancer: A systematic review and meta-analysis of observational studies

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Abstract

BACKGROUND

Personalized nutrition and protective diets and lifestyles represent a key cancer research priority. The association between consumption of specific dietary components and colorectal cancer (CRC) incidence has been evaluated by a number of population-based studies, which have identified certain food items as having protective potential, though the findings have been inconsistent. Herein we present a systematic review and meta-analysis on the potential protective role of five common phytochemically rich dietary components (nuts, cruciferous vegetables, citrus fruits, garlic and tomatoes) in reducing CRC risk.

AIM

To investigate the independent impact of increased intake of specific dietary constituents on CRC risk in the general population.

METHODS

Medline and Embase were systematically searched, from time of database inception to January 31, 2020, for observational studies reporting CRC incidence relative to intake of one or more of nuts, cruciferous vegetables, citrus fruits, garlic and/or tomatoes in the general population. Data were extracted by two independent reviewers and analyzed in accordance with the Meta-analysis of

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Observational Studies in Epidemiology (MOOSE) and Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) reporting guidelines and according to predefined inclusion/exclusion criteria. Effect sizes of studies were pooled using a random-effects model.

RESULTS

Forty-six studies were identified. CRC risk was significantly reduced in patients with higher *vs* lower consumption of cruciferous vegetables [odds ratio (OR) = 0.90; 95% confidence interval (CI): 0.85-0.95; $P < 0.005$], citrus fruits (OR = 0.90; 95%CI: 0.84-0.96; $P < 0.005$), garlic (OR = 0.83; 95%CI: 0.76-0.91; $P < 0.005$) and tomatoes (OR = 0.89; 95%CI: 0.84-0.95; $P < 0.005$). Subgroup analysis showed that this association sustained when looking at case-control studies alone, for all of these four food items, but no significant difference was found in analysis of cohort studies alone. Nut consumption exhibited a similar trend, but overall results were not significant (OR = 0.72; 95%CI: 0.50-1.03; $P < 0.07$; $I^2 = 90.70\%$). Putative anticarcinogenic mechanisms are proposed using gene-set enrichment analysis of gene/protein perturbations caused by active compounds within each food item.

CONCLUSION

Increased cruciferous vegetable, garlic, citrus fruit and tomato consumption are all inversely associated with CRC risk. These findings highlight the potential for developing precision nutrition strategies for CRC prevention.

Key Words: Colorectal cancer; Disease prevention; Diet; Risk; Nutrigenomics; Meta-analysis

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Core Tip: Personalized nutrition and protective diets and lifestyles represent a key cancer research priority. Herein we present a systematic review and meta-analysis on the potential protective role of five common phytochemically rich dietary components (nuts, cruciferous vegetables, citrus fruits, garlic and tomatoes) in reducing colorectal cancer (CRC) risk. Results show that increased dietary consumption of cruciferous vegetables, citrus fruits, garlic and tomatoes is associated with reduced CRC risk. Active components of these may interact with certain genes to exert this anticarcinogenic effect, highlighting the potential for developing precision nutrition strategies for CRC prevention.

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INTRODUCTION

Colorectal cancer (CRC) imposes a major public health burden and is the third most commonly diagnosed cancer worldwide[1]. Incidence varies globally between ethnicities and geographical locations[2] with an increase noted amongst migrants moving from low- to high-risk countries[3]. Likewise, socioeconomic disparities correlate with CRC incidence with higher rates occurring in more deprived regions[4,5]. Large-scale epidemiological studies have served to highlight the impact of potentially modifiable factors such as diet, obesity, physical activity and smoking on CRC incidence[6,7].

Diet is an essential component of health and is known to play a key role in the development of a vast array of diseases, including cancers of the digestive system[3]. CRC is strongly associated with inflammation and oxidative stress, both of which are believed to be highly influenced by diet[8-10]. For many decades, considerable research efforts have been directed towards the identification of food groups which

increase CRC risk, and broadly these have concluded that diets rich in red- and processed-meat, alcohol, saturated fat, refined grains and sugars, increase the risk of developing CRC[11-15]. Likewise, it is believed that a diet rich in fiber, fruits and vegetables can exert a protective effect[12-14].

The Third Expert Report from the World Cancer Research Fund (2018) has emphasized the importance of protective dietary and lifestyle pattern in creating a metabolic state which is more favorable in preventing cancer as a whole through genetic and epigenetic alterations in carcinogenesis, rather than focusing on specific nutrients or food components alone[14]. However, in order to build a holistic protective lifestyle, it remains important to understand which specific foods have the potential to reduce the risk of certain cancers, so as to establish the determinants of external and endogenous challenges contributing to or preventing cancer carcinogenesis. Additionally, in light of the recently published National Institute of Health (NIH) strategic plan, and the call for research into 'precision nutrition' as a potential "holistic approach to developing comprehensive and dynamic nutritional recommendations relevant to both individual and population health"[16], it is vital to explore ways in which specific foods have the potential to beneficially modify cancer incidence and outcomes.

Observational studies have investigated the relationship between CRC incidence and the consumption of certain food items. This study investigated nuts, cruciferous vegetables, citrus fruits, garlic and tomatoes, because these remain amongst those foods with most epidemiological data available. Additionally, Veselkov *et al*[17] have previously uncovered compounds with anticancer properties within these foods by analyzing the genomic effects of the said compounds which may help explain the underlying cause for any protective associations found through observational studies. Thus, food items were included for analysis in circumstances where a substantial number of studies provided comparative outcome data based on consumption levels and fulfilled all inclusion and no exclusion criteria, as outlined in the methods. Although previous meta-analyses have sought to evaluate the impact of some of these food items[18-23], results have been contradictory and inconclusive. Furthermore, to date there has been no comprehensive meta-analysis, including more recent multinational cohort studies, which unifies all five of these food items, assessing all available and recent evidence. To meet this need, we conducted a systematic review of the literature with pooled analysis of data from all eligible observational studies exploring the effect of increased consumption of nuts, cruciferous vegetables, citrus fruits, garlic and tomatoes on CRC risk in the general population. Furthermore, putative anticarcinogenic mechanisms are highlighted through gene-set enrichment analysis (GSEA).

MATERIALS AND METHODS

Search strategy and study selection

This systematic review and meta-analysis was conducted according to Meta-analysis of Observational Studies in Epidemiology guidelines (MOOSE)[24] and Preferred Reporting Items for Systematic Review and Meta-analyses (PRISMA)[25] guidelines. Two reviewers (Borgas P and Mirnezami R) independently conducted a systematic literature search of Medline and Embase, from time of database inception to January 31, 2020. A detailed summary of the search terms used is provided in the Supplementary material, Appendix 1. There were no restrictions to language or study population. Duplicates between databases were removed. Food items were included for analysis in circumstances where a substantial amount of studies provided comparative outcome data based on consumption levels and fulfilled all inclusion and no exclusion criteria as outlined below.

Inclusion criteria comprised: (1) Observational studies (*i.e.*, case-control or cohort); (2) The exposure variable being at least one of the following five dietary components; nuts (including peanuts and peanut butter), cruciferous vegetables (including studies reporting data on individual types of cruciferous vegetables as well as grouped data for several types of Cruciferae), citrus fruits, garlic, tomatoes; (3) Colorectal adenocarcinoma incidence being the outcome (studies investigating benign adenomas or polyps were excluded); (4) Provision of adjusted odds ratios (ORs) or relative risks (RRs) with 95% confidence intervals (CIs); (5) Participants aged ≥ 18 without prevalent cancers at baseline; and (6) Newcastle-Ottawa scale (NOS) > 5 indicating sufficient study quality [26]. A sensitivity analysis was performed to assess the robustness of results with respect to the NOS selection criterion.

Further exclusion criteria comprised: (1) Grouping of the above listed food items with other foods (*e.g.*, nuts often with legumes, cruciferous vegetables often with other vegetables); (2) Where multiple publications from the same study population were found, only the study with the largest case cohort and/or most relevant/recent information was included for analysis; and (3) Non-human research.

Additional criteria such as number of cases, method of outcome assessment and, for case-control studies, method cases and controls selected for, were considered and selected for as part of each studies' individual quality assessment using NOS.

Two authors (Borgas P and Mirnezami R) screened the titles of all identified articles independently, to exclude those that were not relevant to the question at hand, did not meet all inclusion criteria or met at least one exclusion criteria. Abstracts and full texts of potentially relevant remaining articles were then screened for eligibility according to the same principles. Full text articles which met all criteria outlined above were retrieved. Discrepancies were resolved by discussion between the two reviewers (Borgas P and Mirnezami R). Reference lists of all identified articles, and other related review articles, systematic reviews and meta-analyses were hand-searched for additional articles

Data extraction

Two authors (Borgas P and Mirnezami R) independently extracted the following information: name of first author, year of publication, study design, characteristics of study population (age range or mean age, sex, country, specific details on patient demographic), dietary exposure, dietary assessment instrument used, outcomes (including cancer site), comparison, OR or RR (95%CI), adjusted variables, NOS. For case-control studies the following additional information was extracted: number of cases and number of controls. For cohort studies the following additional information was extracted: number of participants at baseline, number of CRC cases, person years, length of follow-up (Supplementary Tables 1 and 2). These variables were judged to be most relevant to the outcome studied. Where multiple estimates for the association of the same outcome were used, the one with the highest number of adjusted variables was extracted.

Quality assessment

The NOS was used to grade the quality and assess confounding of each included study by two independent investigators (Borgas P and Mirnezami R)[26]. Studies scoring less than 6 were excluded in analysis. Disagreements were resolved by discussion.

Statistical analysis

Meta-analyses were conducted in Stata software, version 16.1 (StataCorp LLC). A random-effects model was employed, and the inverse variance method with restricted maximum likelihood was applied to assess the association of intake of each dietary component with the risk of CRC. Between-study heterogeneity was assessed using I^2, in order to define the percentage of variability in effect size estimates which were the result of differences between studies. An $I^2 > 50\%$ suggests high heterogeneity. Subgroup analyses were performed, stratifying by study design to account for the increased risk of biases related to the retrospective assessment of diet in case-control studies compared to cohort studies.

For all studies, we selected the effect- sizes computed by comparing the highest *vs* lowest dietary exposure category and adjusted for confounding variables. For studies reporting gender-specific results only, we included effect size for male and female participants separately and these are therefore displayed as separate studies in the accompanying forest plots, despite originating from the same publication. For studies presenting gender-specific as well as merged male and female data, we included the merged data only. We followed the same procedure for studies reporting data on all CRC types *vs* site-specific CRC. Given that the absolute risk of CRC is low in human, the ORs should approximate the RRs; therefore, we reported all results as ORs for simplicity. Supplementary Table 3 demonstrates the number of studies reporting sub-type specific data based on sex and/or site of cancer for each food item, confirming a relative lack in sufficient numbers of studies reporting such specified data to be able to perform separate analyses for sex and CRC subsite.

Bias analysis

We evaluated the presence of bias in the form of small-study effects using contour-enhanced funnel plots and Egger regression asymmetry testing. For funnel plots, we

used contour lines representing 1%, 5% and 10% significance. We assessed publication bias by visually inspecting the funnel plots, with publication bias being suspected when smaller studies were absent from the non-significant regions. Egger's test was used to obtain a statistical measure of funnel plot asymmetry with the null hypothesis being that the funnel plot is symmetric (*i.e.*, there is no evidence of small-study effects). In this case, a P value > 0.05 implies no evidence of small-study effects.

GSEA

We investigated potential anticarcinogenic mechanisms of food items by building a profile of genomic perturbations caused by each food item and identifying biological pathways affected by these perturbations. We built food profiles of genomic perturbations by averaging genomic perturbations caused by predicted anticarcinogenic compounds within each food as previously proposed by Veselkov *et al*[17]. Pathway analytics were performed using Gene Set Enrichment Analysis v4.0.3, and PANTHER [27]. An extended description of this methodology is provided in Supplementary material, Appendix 2.

RESULTS

Our initial search strategy identified 534, 236, 466, 113 and 370 records on cruciferous vegetables, citrus fruits, garlic, tomatoes and nuts, respectively. Based on our predefined inclusion/exclusion criteria 33, 12, 10, 11 and 7 records, respectively, have been included in this systematic review and meta-analysis. Figure 1 summarizes the study selection process for each dietary component.

Sensitivity analysis

For each of the 5 food items, there was no significant discrepancy between pooled ORs of analyses including all studies, before exclusion based on NOS, compared to analyses after excluding studies with NOS ≤ 5 (Supplementary Table 4). This confirms robustness of the NOS ≤ 5 exclusion criteria used in study selection. All other parameters used in study selection were not arbitrary but rather based on the research question at hand, and thus do not require sensitivity analysis.

Meta-analysis

Pooled ORs for each of the five food items are presented in Table 1. Overall analysis of case-control and cohort studies combined showed that higher dietary intake of cruciferous vegetables, citrus fruits, garlic and tomatoes were all significantly associated with reduced incidence of CRC. Cohort studies on nut consumption showed the same trend.

Cruciferous vegetable intake was associated with a 10% reduction in CRC risk, with low heterogeneity and no evidence of small-study effect (61 studies, 33 publications [28-60]; OR = 0.90; 95%CI: 0.85-0.95; P = 0.00; I^2 = 31.02%, Egger P = 0.57) (Figure 2). Dietary intake of citrus fruits was also associated with a 10% risk reduction in CRC incidence, with low heterogeneity and no evidence of small-study effect (20 studies, 12 publications[33,35,42,48,50,52,53,55,56,61-63]; OR = 0.90; 95%CI: 0.84-0.96; P = 0.00; I^2 = 21.65%, Egger P = 0.46) (Figure 3). Higher garlic intake was associated with a 17% reduction in risk of CRC with low heterogeneity and no evidence of small-study effect (14 studies, 10 publications[33,42,48,58,60,61,64-67]; OR = 0.83; 95%CI: 0.76-0.91; P = 0.00; I^2 = 32.64%, Egger P = 0.11) (Figure 4). Higher dietary tomato intake was associated with an 11% reduction in CRC risk, with low heterogeneity (16 studies, 11 publications[33,34,42-44,48,51,54,63,68,69]; OR = 0.89; 95%CI: 0.84-0.95; P = 0.00; I^2 = 0%, Egger P = 0.02) (Figure 5). However, the P value acquired with Egger's regression indicates that we could not discard the presence of small-study effects.

The association of increased dietary intake of all four food items with reduced CRC risk, is maintained in subgroup analysis of case-control studies alone, however cohort studies alone show an association which does not reach significance for all four aforementioned food items (Table 1).

Overall analysis of both case-control and cohort studies investigating higher dietary nut consumption demonstrated no statistically significant association with reduced risk of CRC, and significant heterogeneity was observed between studies (9 studies, 7 publications[28,40,49,70-73]; OR = 0.72; 95%CI: 0.50-1.03; P = 0.07; I^2 = 91.85%, Egger P = 0.21) (Figure 6). However, subgroup analysis of cohort and case-control studies separately showed a significant association between nut consumption and reduced CRC risk for cohort studies with low heterogeneity (4 studies, 3 publications[49,71,73];

Table 1 Association between dietary components and colorectal cancer risk

Dietary component		Number of studies (number of publications)[1]	Pooled OR (95%CI)	*P* value	Heterogeneity [I^2 (%)]	Egger *P* value
Cruciferous vegetables	All	61 (33)	0.90 (0.85-0.95)	0.00	31.02	0.58
	Case-control	38 (21)	0.85 (0.78-0.93)	0.00	41.94	
	Cohort	23 (12)	0.94 (0.89-1.00)	0.07	0.00	
Citrus fruits	All	20 (12)	0.90 (0.84-0.96)	0.00	21.65	0.46
	Case-control	9 (7)	0.94 (0.85-1.04)	0.00	42.81	
	Cohort	11 (5)	0.86 (0.77-0.95)	0.26	0.00	
Garlic	All	14 (10)	0.83 (0.76-0.91)	0.00	32.64	0.12
	Case-control	8 (6)	0.92 (0.72-1.18)	0.00	47.80	
	Cohort	6 (4)	0.81 (0.72-0.90)	0.51	35.38	
Tomatoes	All	16 (11)	0.89 (0.84-0.95)	0.00	0.00	0.02
	Case-control	13 (9)	0.97 (0.84-0.95)	0.00	0.00	
	Cohort	3 (2)	0.87 (0.8-0.93)	0.68	0.00	
Nuts	All	9 (7)	0.72 (0.50-1.03)	0.07	91.85	0.22
	Case-control	5 (4)	0.74 (0.39-1.43)	0.38	94.35	
	Cohort	4 (3)	0.74 (0.58-0.94)	0.01	35.48	

[1]Where studies reported gender-specific or cancer site-specific results only rather than combining these into one result, we included effect size for male and female participants separately and likewise site-specific cancer data separately, and these are therefore displayed as 2 separate studies in the accompanying forest plots, despite originating from the same publication. CI: Confidence interval; OR: Odds ratio.

OR = 0.74; 95%CI: 0.58-0.94; P = 0.01; I^2 = 35.48%). Case-control studies showed no significant association with high heterogeneity (5 studies, 4 publications[28,40,70,72]; OR 0.74; 95%CI: 0.39-1.43; P = 0.38; I^2 = 94.35%).

Bias analysis

Funnel plots of citrus fruits and cruciferous vegetables show a clear symmetrical distribution of studies, with both large and small-sized studies in the significant and non-significant regions. Symmetrical distribution is not evident in funnel plots generated for garlic, nuts and tomato. The P values for Egger's test can be found in Table 1. As expected, tests of symmetry for cruciferous vegetable and citrus fruit studies demonstrated robust P values (0.58 and 0.46, respectively); garlic and nuts studies had more modest but still non-significant P values (0.12 and 0.22 respectively); the P value for tomato studies was 0.02. Funnel plots of all food items can be found in Supplementary Figures 1-5. These analyses suggest that there is no evidence of small study effects (publication bias) in studies evaluating cruciferous vegetables, citrus fruits, garlic, and nuts; whereas this possibility cannot be discarded for studies exploring tomato intake in relation to CRC incidence.

GSEA

A list of the genes and proteins most affected by active compounds within each food item (Supplementary Tables 5 and 6) was subjected to pathway analytics using gene-set enrichment analysis (see Methods and Supplementary material, Appendix 2). The most affected genes and pathways are presented in Figure 7. The node colour represents shared biological pathway functionality or entity category (food compounds, food genomic perturbation profile). Among the top perturbed pathways were cell cycle, apoptosis, p53 signalling, and the MAPK signalling pathway, as well as other colorectal-cancer-specific pathways. Supplementary Table 6 contains a full list of perturbed genes/pathways for each food item.

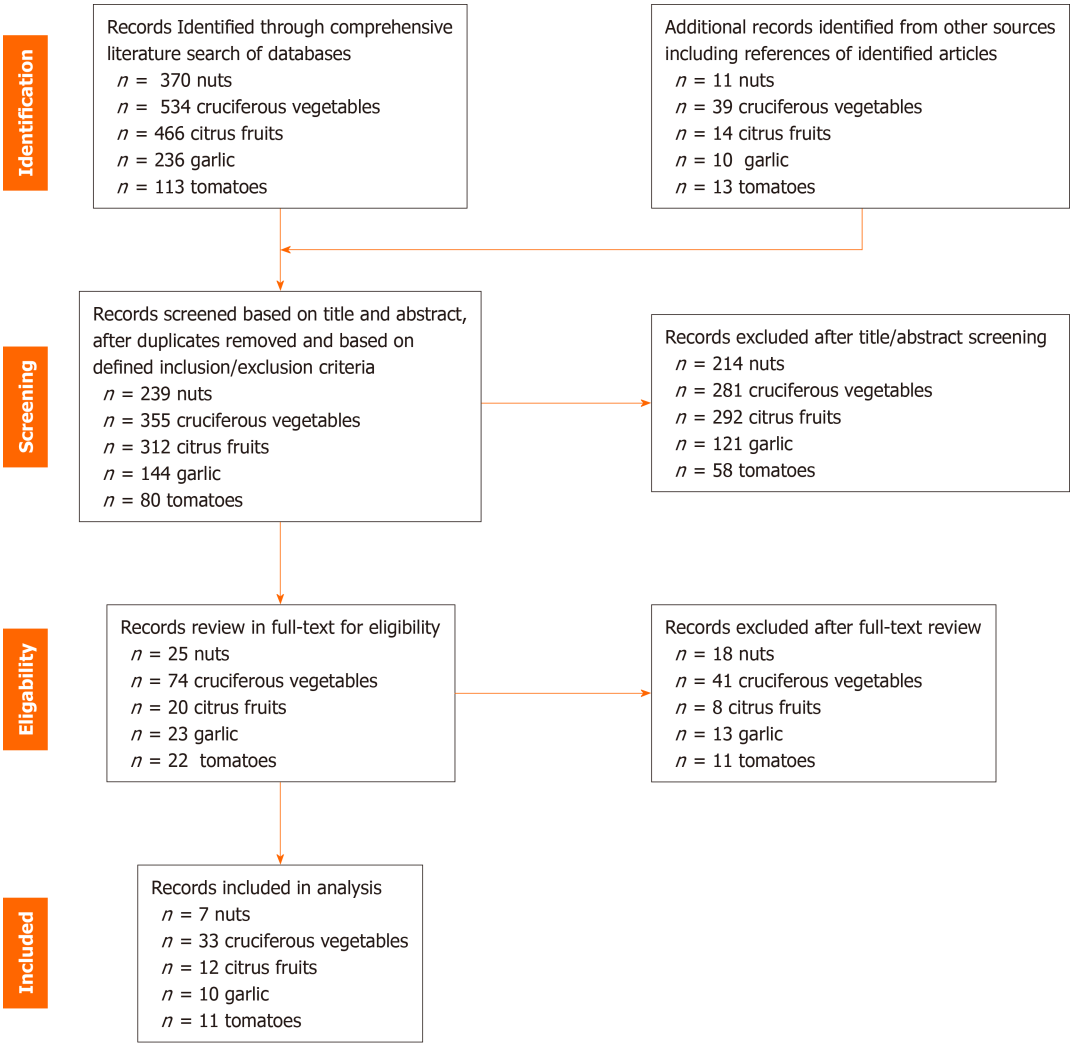


Figure 1 Flow Diagram summarizing systematic literature search strategy and outcomes.

DISCUSSION

Dietary chemoprevention is emerging as a potentially cost effective and innovative way to prevent and treat cancer and has been highlighted as a key research priority by the NIH. The importance of this in implementing a holistic protective lifestyle is also highlighted in the Third Expert Report from the WCRF (World Cancer Research Fund) [14,16]. The findings of this meta-analysis indicate that higher dietary intake of the phytochemically rich cruciferous vegetables, citrus fruits, garlic and tomatoes is associated with a significant reduction in the risk of CRC, suggesting that promoting increased consumption of these foods should be considered as part of a holistic CRC protective diet. Meta-analysis of cohort studies investigating increased nut consumption showed the same significant association. Compared to existing systematic reviews and meta-analyses which have only explored the effect of single dietary components on CRC or have largely focused on dietary components associated with increased CRC risk[18-23], this is the first study to investigate the protective potential of these five highly investigated dietary components on CRC incidence. Additionally, to the authors' knowledge, data on increased dietary intake of tomatoes and citrus fruits and CRC risk have not previously been subjected to meta-analytical evaluation.

Combined analysis of case-control and cohort studies showed significant results overall, however sub-group analyses revealed that the results are significant for case-control but not cohort studies. Although the Third Expert Report published by the WCRF has highlighted the importance of focusing evidence on cohort studies, there are a number of factors which make the evidence from cohort studies more difficult to interpret and subject to bias. Case-control studies remain a stronger source of evidence for diseases with long latency periods including CRC for which the incidence increases

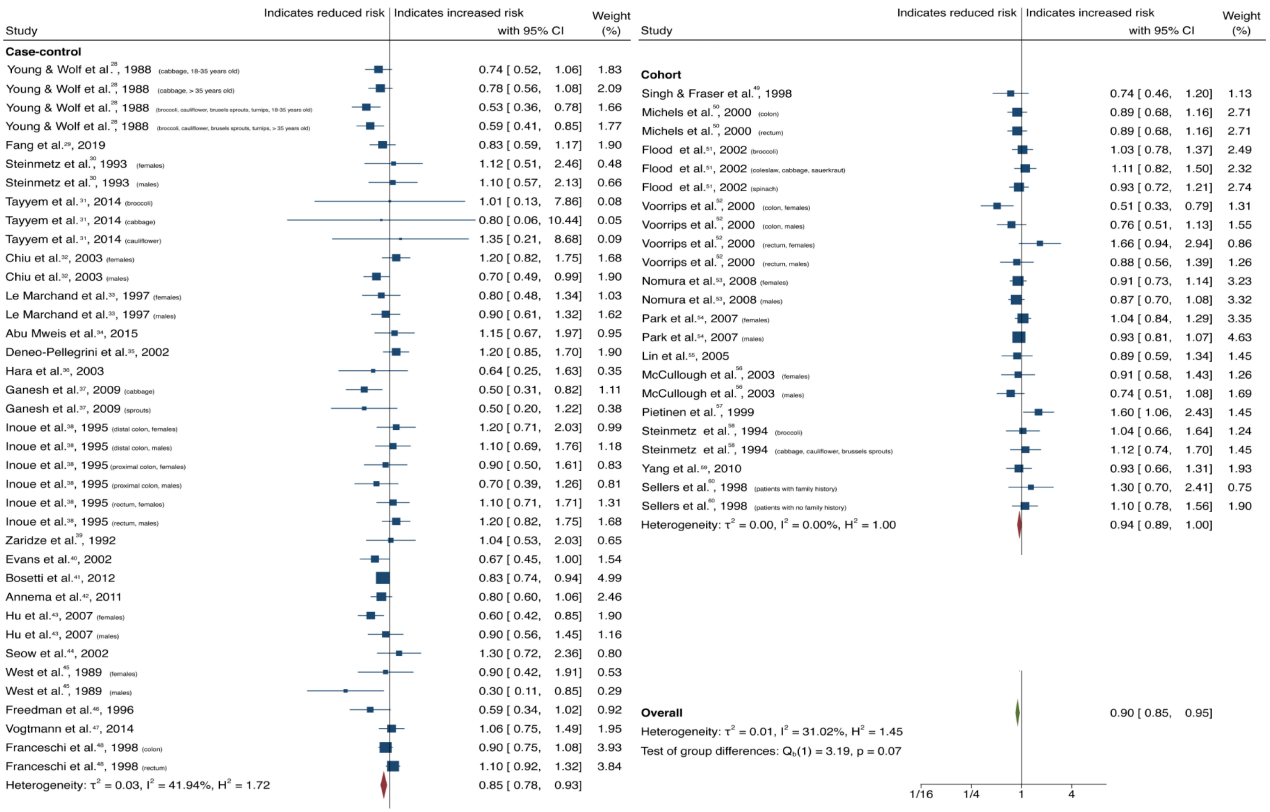


Figure 2 Forest plot showing the association between dietary cruciferous vegetable consumption and colorectal cancer risk. Squares and horizontal lines represent study-specific odds ratios (ORs) and 95% confidence intervals (CIs). The size of each square indicates the weighting of the study in the overall analysis depending on population size. Diamonds represent pooled ORs of all studies of that food item and 95%CI of the overall population. Vertical line indicates OR 1.0. CI: Confidence interval.

with age in both men and women. Thus, evidence from cohort studies is stronger if patients are closer to this age group and with longer follow-up period. However, many of the studies included patients as young as approximately 30 or 45 years old[49,50,53,55,59,66,71] and the average years of follow up of all cohort studies in this analysis was 9.4 years with certain studies having follow-up periods as short as 3-6 years[49,54,58,65,66]. Only 3 out of a total of 16 identified cohort studies followed patients up for 10 or more years[50,71,73]. This could lead to significant amounts of bias which is likely to be a major contributing factor as to why the association observed was not significant when analyzing cohort studies alone. For this reason, emphasis in this meta-analysis was not set solely on cohort studies alone but case-control studies were also viewed as highly informative and valuable in analysis despite the potential for biases related to retrospective assessment of diet in case-control studies. Hence, and in view of both types of epidemiological studies having the potential for different types of bias, it was thought to be most accurate to place most emphasis on the overall results in combination rather than focusing on the results of the sub-type analysis alone.

Despite an overall protective correlation having been observed for these foods, variability between outcomes of individual studies is evident, which may be attributable to a number of factors. Firstly, there is much variation in highest *vs* lower intake comparisons between studies with some not providing numerical data on intake servings, making a continuous dose-response analysis not feasible. Additionally, differences in geographical location may also explain variability in results between individual studies as cultural differences in dietary and lifestyle patterns as a whole may influence any protective effects observed. Although only studies which provided adjusted OR and RR data were included in analysis, there was a degree of variance in stratified analysis between studies and the extent to which confounders and other lifestyle factors were adjusted for between studies was inconsistent.

Significant divergences of individual studies from the mean may be attributable to study design and quality. For example, Levi *et al*[61], investigating citrus fruits and garlic, included much younger patients (from 27 years) compared to the majority of remaining studies which had an approximate age range between 50-80 years. Others,

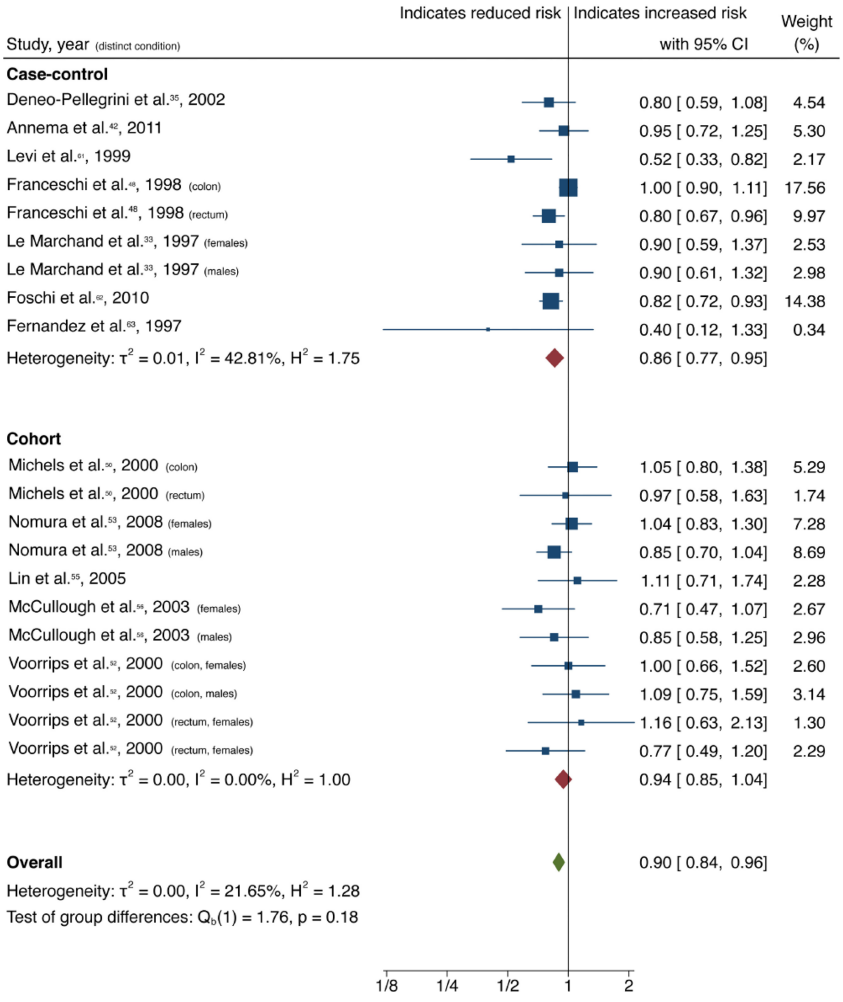


Figure 3 Forest plot showing the association between dietary citrus fruit consumption and colorectal cancer risk. Squares and horizontal lines represent study-specific odds ratios (ORs) and 95% confidence intervals (CIs). The size of each square indicates the weighting of the study in the overall analysis depending on population size. Diamonds represent pooled ORs of all studies of that food item and 95%CI of the overall population. Vertical line indicates OR 1.0. CI: Confidence interval.

like Fernandez *et al*[63], investigating citrus fruits and tomatoes, did not adjust for as many significant variables like smoking, alcohol, total energy intake, education, consumption of other foods, as most other studies. These factors may have contributed to increased variability demonstrated in wide CIs and could explain the reason for the risk association to appear much stronger in these studies compared to the overall result. Conversely, some studies included a relatively low number of participants[31,36,39,44,63,72], participants selected from a defined population group attributing to selection bias[57], did not report details of their study design leading to low NOS[44] or studied specific individual components of the overall food items investigated here (*e.g.*, single types of cruciferous vegetables)[31]. All such factors lead to reduced reliability of the studies' results which is evidenced in the analysis by a low weight in random-effects model. Nevertheless, all studies scored > 5 on the quality assessment criteria using NOS and did not meet any other exclusion criteria and hence remain valuable contributors to the overall analysis.

Although the exact mechanisms for the associations reported here remain unclear, all four dietary components are rich in compounds believed to exert anticarcinogenic effect. To investigate these putative mechanisms further, we compiled a list of 'anti-carcinogenic' compounds found in each of the food items studied, based on data previously published by Veselkov *et al*[17] (see Supplementary Table 5). We performed further pathway enrichment analyses of genes with which these anticarcinogenic compounds are known to interact, as per the methodology described by Veselkov *et al*[17] (see Supplementary material, Appendix 2).

Garlic contains five anticarcinogenic compounds, belonging to organosulfur (ajoene, Di-2-propenyl sulphide), flavonoid (apigenin, quercetin) and phenol (phloroglucinol) categories. Several of the top 20 genes perturbed by these compounds take part in

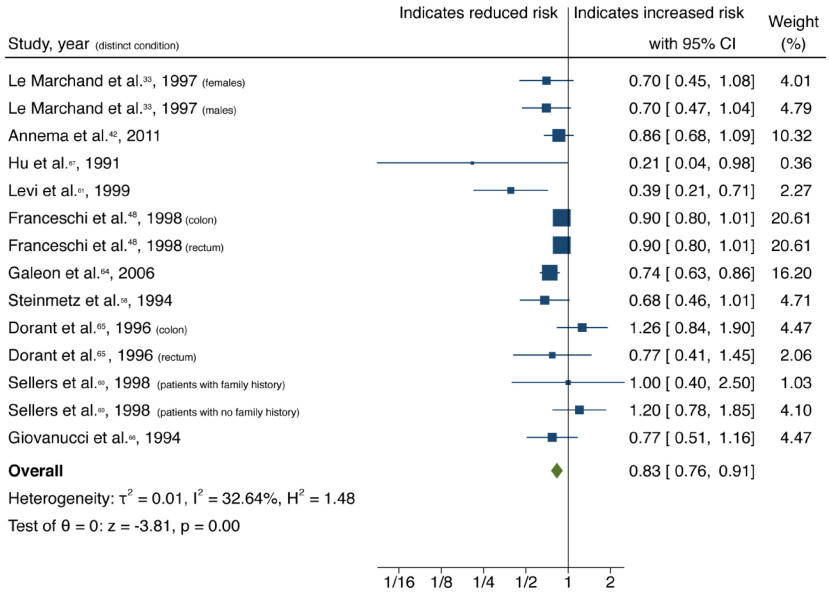


Figure 4 Forest plot showing the association between dietary garlic consumption and colorectal cancer risk. Squares and horizontal lines represent study-specific odds ratios (ORs) and 95% confidence intervals (CIs). The size of each square indicates the weighting of the study in the overall analysis depending on population size. Diamonds represent pooled ORs of all studies of that food item and 95%CI of the overall population. Vertical line indicates OR 1.0. CI: Confidence interval.

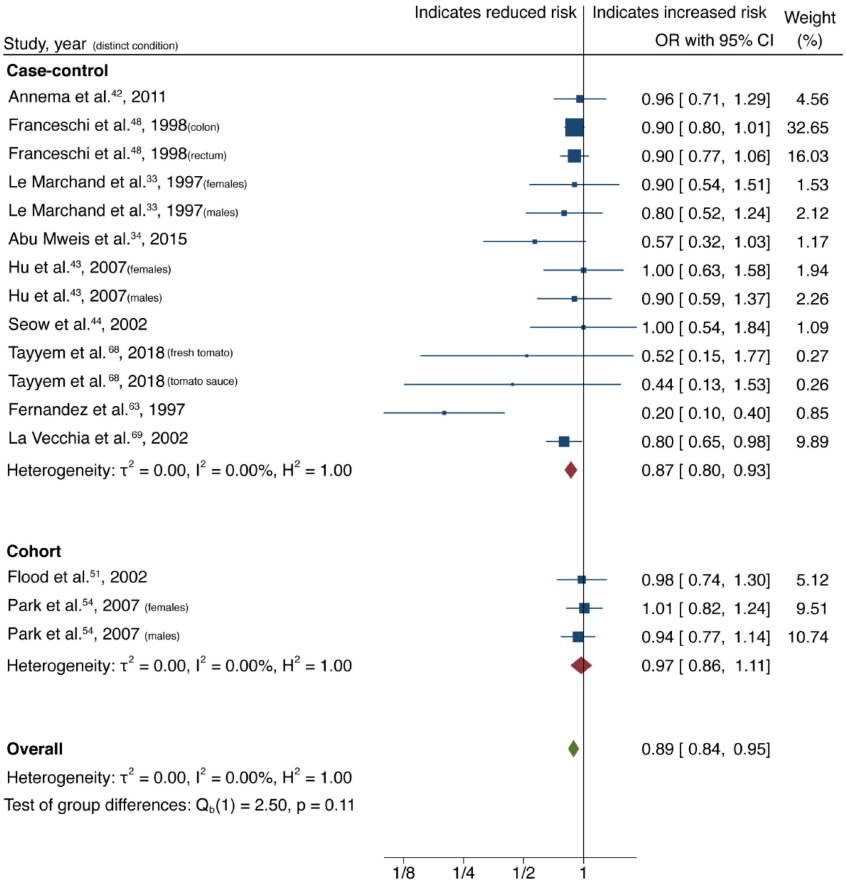


Figure 5 Forest plot showing the association between dietary tomato consumption and colorectal cancer risk. Squares and horizontal lines represent study-specific odds ratios (ORs) and 95% confidence intervals (CIs). The size of each square indicates the weighting of the study in the overall analysis depending on population size. Diamonds represent pooled ORs of all studies of that food item and 95%CI of the overall population. Vertical line indicates OR 1.0. OR: Odds ratio; CI: Confidence interval.

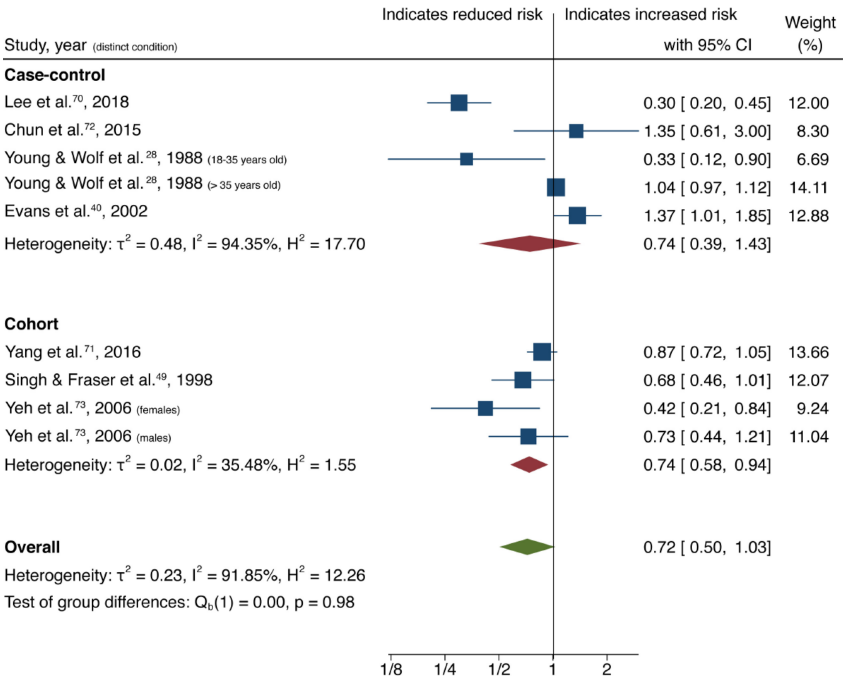


Figure 6 Forest plot showing the association between dietary nut consumption and colorectal cancer risk. Squares and horizontal lines represent study-specific odds ratios (ORs) and 95% confidence intervals (CIs). The size of each square indicates the weighting of the study in the overall analysis depending on population size. Diamonds represent pooled ORs of all studies of that food item and 95%CI of the overall population. Vertical line indicates OR 1.0. CI: Confidence interval.

pathways related to inflammation, apoptosis, and angiogenesis. Furthermore, organosulfur and flavonoid compounds are thought to have chemoprotective properties[74,75] through a number of mechanisms including reduction of aberrant crypt formation [76], antioxidant effects, regulation of cell cycle arrest, induction of apoptosis, and activation of metabolizing enzymes which detoxify carcinogens and inhibit angiogenesis[77,78].

In addition to flavonoids (quercetin), tomatoes contain anticarcinogenic compounds including prenol lipids (prolycopene, lupeol), progesterone and carbohydrate (ferulic acid 4-glucoside). Citrus fruits are also rich in flavonoids (luteolin 7-rhamnosyl-glucoside, quercetin, tetramethylquercetin, didymin, quercetagetin) and prenol lipids (carvone, obacunon, umbelliprenin) and additionally contain steroids (brassinolide) and carbohydrates (xyan). The genes targeted by these anticarcinogenic compounds were also predominantly found to be involved in apoptosis, angiogenesis and inflammation. GSEA revealed interaction with MAPK and p53 signalling pathways, which are key drivers of CRC development and progression[79,80].

Cruciferous vegetables contain nine anticarcinogenic compounds including flavonoids (quercetin), and prenol lipids (carvone, gibberellin A116) but also isothiocyanates (erucin), indoles (1H-Indole-3-methanol, brassinin), allyl sulphur compounds (di-2-propenyl sulfide), organooxygen compounds (4-methoxyglucobrassicin) and steroids (brassinolide). Glucosinolates are precursors of isothiocyanates and indole-3-carbinol[81,82], which inhibit carcinogen-activating enzyme and angiogenesis, and detoxify carcinogens, inducing apoptosis and preventing cell cycle progression[44,83]. Animal studies have shown that cruciferous vegetables activate phase I (cytochrome P450) and II (glutathionin S-transferase) cell-cycle enzymes, and that this joint induction leads to a favourable metabolic profile, aiding in the elimination of chemical carcinogens by interacting with isothocyanate metabolism[44,59,81-83].

Nuts contain macro- and micro-nutrients including fibre, vitamins (BE, folate, niacin), minerals (zinc, potassium, calcium, magnesium) polyphenols, folate, phytoestrogens, phytochemicals (flavonoids, carotenoid and phytosteroils) and other bioactive substances. Additional anticarcinogenic compounds, as confirmed through our analysis, also include unsaturated fatty acids (dihydroxystearic acid), flavonoids (procyanidin B3 procyanidin B2, quercetin), garlic acid, naphthalene (plumbagin) prenol lipids (betulinic acid) and hydorcycoumarins (aesculetin), all of which harbour anti-inflammatory properties[84-86]. They display anticarcinogenic effect by reducing DNA damage and regulating immunological activity and inflammatory responses[84,87]. In vitro studies have demonstrated that fermented nuts exhibit chemopreventative

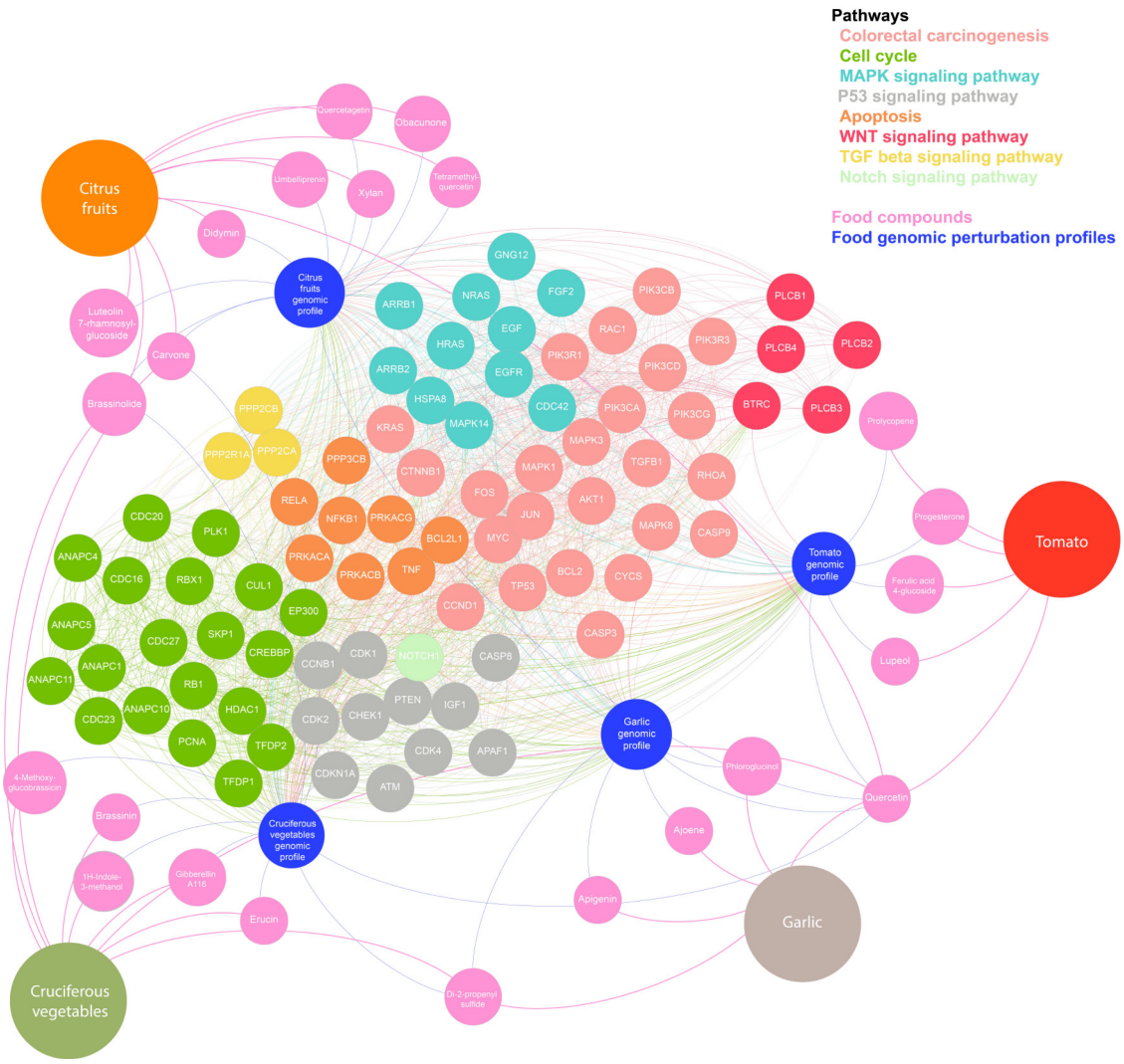


Figure 7 Top genes and pathways perturbed by food items. Each food item is connected to the active compounds found commonly within that food. Food genomic profiles are connected to genes perturbed. Node colour represents shared biological pathway functionality or entity category (food compounds, food genomic perturbation profile).

effects by decreasing tumour-promoting deoxycholic acid, increasing short-chain fatty acids and protecting against oxidative stress[88]. Our results showed a significant association between higher dietary nut intake, and reduced CRC risk, supporting the findings of a previous meta-analysis[18]. However significant heterogeneity was observed meaning that a conclusion cannot reliably be reached based on currently available data.

In addition to these postulated mechanisms specific to each food items' associated bioactive compounds, pathway enrichment analysis of genomic perturbation profiles of all food items showed an over-representation of several pathways known to be linked with CRC pathogenesis including the MAPK, EGFR, PI3K/AKT, TGF-b and Wnt signalling pathways[89]. This implies that the active compounds present in the five food items studied, interact with genes and pathways involved in the development of CRC. This in turn suggests that the protective influence of these dietary components, as demonstrated by this meta-analysis, may be directly attributable to novel nutrigenomic interactions.

The findings presented herein support the vision recently outlined in the NIH strategic plan, which highlights an urgent need to accelerate nutrition research over the next decade and demonstrate the potentially important role that precision nutrition can play in the prevention and treatment of CRC. These findings should form the foundation on which to design randomized clinical trials for the evaluation of food-based approaches in modifying CRC risk. In the future, we envisage that those at risk of CRC could have personalized 'food passports' developed by care providers, specifically composed of food plans to build dietary patterns designed to reduce their risk of cancer. Future research should focus on understanding the underlying

mechanisms behind the observed effects and exploring the potential interactions between individual genes/pathways and active compounds of the investigated foods in more detail. Furthermore, the nature of the observed findings should be explored using linear and continuous exposure analyses to identify thresholds and plateaus in the protective effect and investigate dose-response effects of consumption of the foods evaluated. Lastly, the influence of food cultivation, packaging, storage and preparation methods should be explored, all of which may influence the associations observed.

CONCLUSION

This meta-analysis demonstrates that an increased consumption of cruciferous vegetables, citrus fruits, garlic and tomatoes is associated with a reduction in the incidence of CRC. These findings support the rapidly expanding initiative for exploring the role of nutrition in the prevention and treatment of cancer and could pave the way for personalized CRC protective diets in the future.

ARTICLE HIGHLIGHTS

Research background

Personalized nutrition and protective diets and lifestyles represent a key cancer research priority according to The Third Expert Report from the World Cancer Research Fund (2018) and the recently published National Institute of Health strategic plan. The association between consumption of specific dietary components and colorectal cancer (CRC) incidence has been evaluated by a number of large-scale, population-based, epidemiological studies, which have identified certain food items as having protective potential, though the findings have been inconsistent.

Research motivation

Although previous meta-analyses have sought to evaluate the impact of some of these food items, results have been contradictory and inconclusive. To date there has been no comprehensive meta-analysis, including more recent multinational cohort studies, which unifies all five of these food items, assessing all available and recent evidence. Furthermore, anticancer properties could be explained by genomic effects of certain compounds contained in these phytochemically rich foods. This may help explain the underlying cause for any protective associations. Thus, we present a systematic review and meta-analysis on the potential protective role of five common phytochemically rich dietary components (nuts, cruciferous vegetables, citrus fruits, garlic and tomatoes) in reducing CRC risk. Putative anticarcinogenic mechanisms are highlighted through gene-set enrichment analysis.

Research objectives

To investigate the independent impact of increased intake of five dietary constituents (nuts, cruciferous vegetables, citrus fruits, garlic and tomatoes) on CRC risk in the general population.

Research methods

Medline and Embase were systematically searched, from time of database inception to January 31, 2020, for observational studies reporting CRC incidence relative to intake of one or more of nuts, cruciferous vegetables, citrus fruits, garlic and/or tomatoes in the general population. Data were extracted by two independent reviewers and analyzed in accordance with the Meta-analysis of Observational Studies in Epidemiology (MOOSE) and Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) reporting guidelines and according to predefined inclusion/exclusion criteria. Effect sizes of studies were pooled using a random-effects model. Using gene set enrichment analysis, a profile of genomic perturbations caused by each food item was built and biological pathways affected by these perturbations were identified.

Research results

This systematic review and meta-analysis of 46 observational studies found that increased dietary consumption of cruciferous vegetables, citrus fruits, garlic and

tomatoes is associated with a significant reduction in the risk of developing CRC. Consumption of nuts exhibited a similar trend, but results were not significant. The underlying mechanisms of the observed effects remain unclear, although putative anticarcinogenic mechanisms are proposed using the results of gene-set enrichment analysis of gene-protein perturbations caused by active compounds within each food item.

Research conclusions

Increased cruciferous vegetable, garlic, citrus fruit and tomato consumption are all inversely associated with CRC risk. These findings highlight the potential for precision nutrition strategies and development of personalized food maps in CRC prevention for both the general population and at-risk individuals.

Research perspectives

The findings should stimulate future research to focus on understanding the nature of the observed effects, by exploring the epigenetic interactions of active compounds within the investigated foods. Additionally, linear and continuous exposure analyses should be explored to identify consumption thresholds for the observed effects. Results should then encourage the design of randomized controlled trials to assess food-based approached in modifying CRC risk.

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CASE REPORT

Long-term complete response in metastatic poorly-differentiated neuroendocrine rectal carcinoma with a multimodal approach: A case report

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Abstract

BACKGROUND

Neuroendocrine gastrointestinal tumors (NETs) are rare and have different natural behaviors. Surgery is the gold standard treatment for local disease while radiotherapy has been demonstrated to be ineffective. Poorly differentiated neuroendocrine carcinomas (NECs) represent only 5%-10% of digestive NETS. Due to aggressive growth and rapid metastatic diffusion, early diagnosis and a multidisciplinary approach are mandatory. The role of surgery and radiotherapy in this setting is still debated, and chemotherapy remains the treatment of choice.

CASE SUMMARY

A 42-year-old male with an ulcerated bleeding rectal lesion was diagnosed with a NEC G3 (Ki67 index > 90%) on May 2015 and initially treated with 3 cycles of first-line chemotherapy, but showed early local progressive disease at 3 mo and underwent sphincter-sparing open anterior low rectal resection. In September 2015, the first post-surgery total-body computed tomography (CT) scan showed an early pelvic disease relapse. Therefore, systemic chemotherapy with FOLFIRI was started and the patient obtained only a partial response. This was followed by pelvic radiotherapy (50 Gy). On April 2016, a CT scan and 18F-fluorodeoxy-

glucose positron emission tomography imaging showed a complete response (CR) of the pelvic lesion, but pathological abdominal inter-aortocaval lymph nodes were observed. Due to disease progression of abdominal malignant nodes, the patient received radiotherapy at 45 Gy, and finally obtained a CR. As of January 2021, the patient has no symptoms of relapse and no late toxicity after chemotherapy or radiotherapy.

CONCLUSION
This case demonstrates how a multimodal approach can be successful in obtaining long-term CR in metastatic sites in patients with high grade digestive NECs.

Key Words: Neuroendocrine tumor; Multimodal therapy; Rectal carcinoma; Surgery; Radiotherapy; Case report

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Core Tip: Neuroendocrine gastrointestinal tumors are rare with different clinical behaviors. Prognostic factors are tumor differentiation, Ki-67 index and the presence of distant metastases. Most of these tumors are well differentiated neuroendocrine tumors with a good prognosis and a 5-year overall survival of 60%-90%. Surgery is the gold standard treatment in local and metastatic disease, while radiotherapy is ineffective. Poorly differentiated neuroendocrine carcinomas represent only 5%-10% of digestive neuroendocrine gastrointestinal tumors. Here we describe the rare case of a patient diagnosed with poorly differentiated rectal metastatic neuroendocrine carcinoma treated with a different treatment approach and with complete remission after 5 years.

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INTRODUCTION

Neuroendocrine gastrointestinal tumors (NETs) are rare and have different natural behaviors. Prognostic factors are tumor differentiation, Ki-67 index and the presence of distant metastases. Most of these tumors are well differentiated NETs[1]; even in the presence of metastasis their prognosis is good with a 5-year overall survival (OS) of 60%-90%. Surgery is the gold standard treatment for local disease and often in metastatic cases, while radiotherapy has been demonstrated to be ineffective[2]. Poorly differentiated neuroendocrine carcinomas (NECs) represent only 5%-10% of digestive NETs and among these rectal NECs account for less than 1% of colorectal malignancies. Relative survival at 5 years is 16.3% overall and 57.4%, 56.4%, 26.3%, and 3.0% for stages I, II, III, and IV cancer[3]. Due to aggressive growth and rapid metastatic diffusion, early diagnosis and a multidisciplinary approach are mandatory. The role of surgery and radiotherapy is still debated, and chemotherapy is the main treatment choice[4]. Based on these data, long-term survivors are rarely described in the literature in the metastatic setting. Here we report a patient with metastatic rectal NEC who maintained a complete response (CR) after 5 years using a different treatment approach.

CASE PRESENTATION

Chief complaints

On May 2015, a 42-year-old male attended our Emergency Department due to acute rectal bleeding.

conflicts of interest.

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History of present illness

An urgent colonoscopy showed the presence of an ulcerated lesion 12 cm distant from the anal verge (Figure 1). Endoscopic histological sampling revealed a NEC G3 (Ki67 index > 90%).

History of past illness

He had no previous health problems, only an allergy to nickel.

Personal and family history

No familial history of gastrointestinal disease or tumors.

Physical examination

On presentation, his vital signs were temperature of 36.5°C, pulse rate of 115 bpm, respiratory rate of 17 breaths/min, blood pressure of 110/60 mmHg, and oxygen saturation of 95%. On general physical examination he looked pale. Abdominal examination revealed a non-distended abdomen. Cardiovascular, pulmonary and neurological examinations were unremarkable.

Laboratory examinations

Complete blood count analysis revealed a huge decrease in hemoglobin level (9.8 mg/mL) and decreased iron and ferritin levels (20 μg/mL and 4 μg/mL, respectively).

Imaging examinations

Magnetic resonance imaging (MRI), computed tomography (CT) scan and 18F-fluorodeoxyglucose positron emission tomography/CT (18F-FDG PET/CT) scan confirmed rectal localization with loco-regional lymph node involvement and the absence of metastatic disease. After 3 cycles of first-line chemotherapy with cisplatinum (CDDP 75 mg/m^2 on day 1, every 3 wk) and etoposide (VP16 100 mg/m^2 on days 1-3, every 3 wk), local progressive disease was shown by MRI and colonoscopy. Local symptoms rapidly worsened, with pain and bleeding.

MULTIDISCIPLINARY EXPERT CONSULTATION

After a multidisciplinary evaluation, surgery was recommended.

FINAL DIAGNOSIS

The histopathology report confirmed small-cell poorly-differentiated NEC pT3N1 (6/14), G3, Ki67 98%, with negative surgical margins.

TREATMENT

In August 2015, the patient underwent sphincter-sparing open anterior low rectal resection, and fashioning of a colonic terminal stoma.

OUTCOME AND FOLLOW-UP

In September 2015, the first post-surgery total-body CT scan showed early disease relapse with a 85 mm pelvic lesion (Figure 2), without any other disease on 18F-FDG PET/CT. Thus, systemic chemotherapy with FOLFIRI (irinotecan 180 mg/m^2 on day 1, followed by folinic acid 400 mg/m^2, a 5-FU bolus of 400 mg/m^2 and 5-FU 1200 mg/m^2 in a 44-h infusion days (1-2 cycles every 14 d), was administered, and a partial response (PR) was obtained after 12 cycles. This was followed by pelvic radiotherapy (50 Gy). On April 2016, CT scan and 18F-FDG PET/CT imaging showed CR of the pelvic lesion, but pathological abdominal inter-aortocaval lymph nodes (Figure 3 and 4). Due to early progression after systemic treatment of abdominal malignant nodes, the patient received radiotherapy at 45 Gy and obtained a CR. He was followed-up with CT scan and 18F-FDG PET/CT every 6 mo. At the last follow-up in January 2021

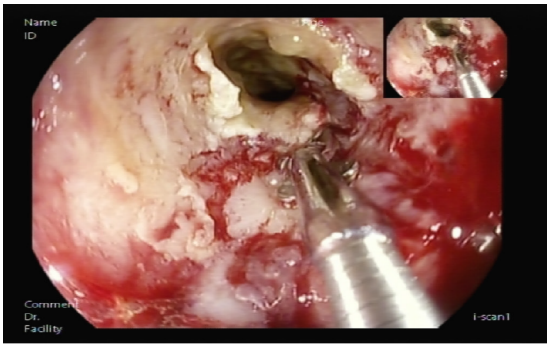


Figure 1 Endoscopic view of rectal neuroendocrine carcinoma.

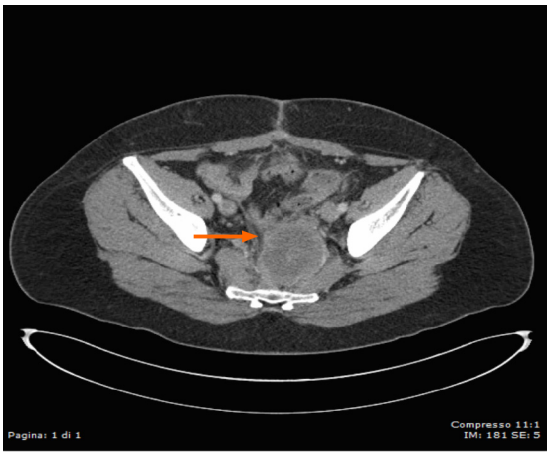


Figure 2 Computed tomography scan view of rectal neuroendocrine carcinoma.

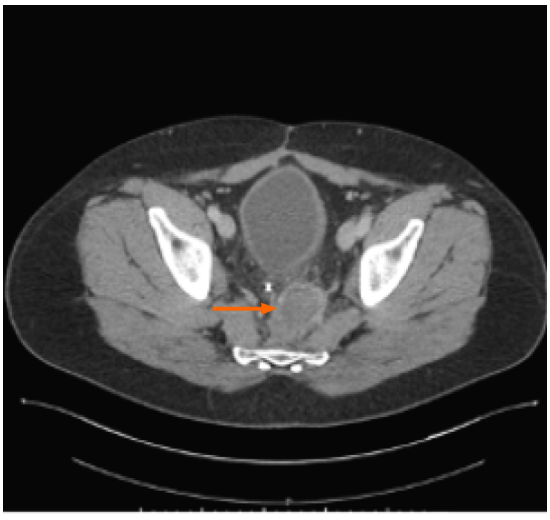


Figure 3 Computed tomography image of rectal lesion downstaging after chemotherapy.

both examinations confirmed CR in the pelvic and metastatic disease (Figure 5). He had no symptoms and no late toxicity after chemotherapy or radiotherapy.

DISCUSSION

Rectal NECs are rare entities and there is no consensus on their treatment especially local disease[5]. As there are no guidelines based on prospective studies, oncologists

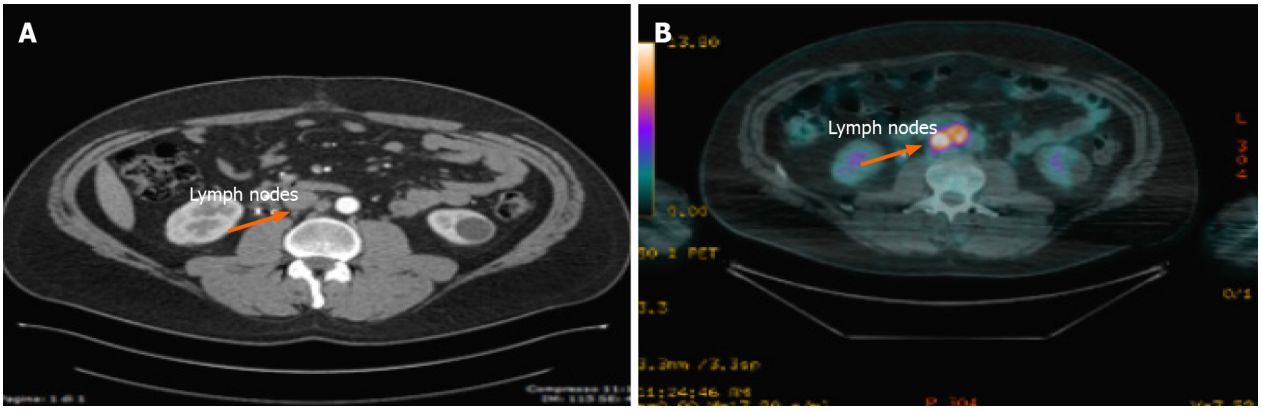


Figure 4 Positron emission tomography/computed tomography images of periaortic lymph nodes. A: Positron emission tomography image; B: Computed tomography image.

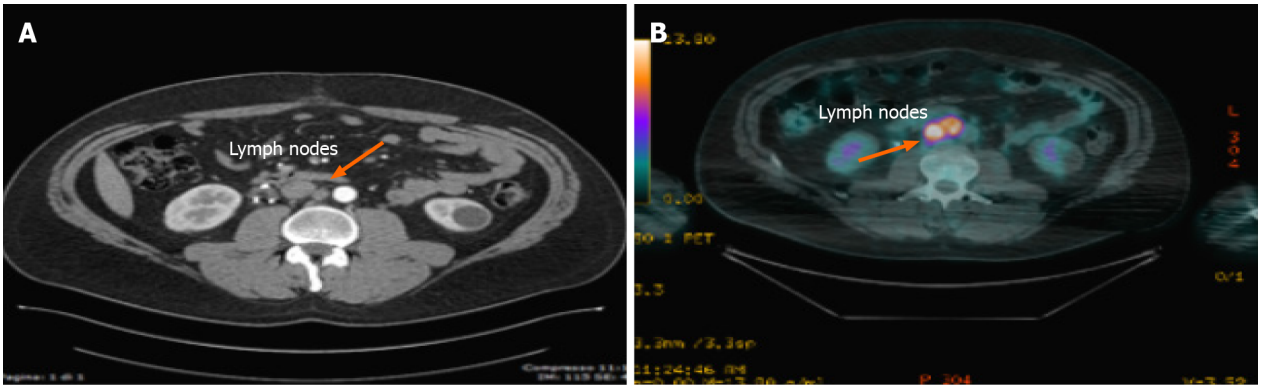


Figure 5 Positron emission tomography/computed tomography images showing complete response after radiotherapy. A: Positron emission tomography image; B: Computed tomography image.

normally use modalities extrapolated from colonic adenocarcinomas or small-cell lung cancer or based on retrospective analysis[6]. The efficacy of systemic chemotherapy for treating digestive metastatic NEC has been largely demonstrated and the role of radiation therapy (RT) and/or surgery is still debated, both in the early stage and metastatic disease[7]. Our case highlights several issues related to the possible clinical treatment of rectal NECs, especially how to treat local primary tumors in patients without metastatic disease. Some retrospective studies agree that there are no differences between surgery and chemoradiotherapy in terms of progression-free survival and OS in local rectal NECs, with disease control in approximately 93% of patients treated exclusively with chemoradiotherapy[8]. These results and the knowledge of high risk of metastatic dissemination for NECs suggest that surgery is not indicated. Moreover, if resection is performed, it has been demonstrated that neoadjuvant, pre-surgery or adjuvant chemotherapy administration is associated with improved rates of survival[9]. As in our case, probably resection is justified in the case of symptomatic or relapse disease.

In the metastatic setting, the gold standard first-line treatment is the combination of platinum and etoposide[10]. Even if there is a high response rate (60%) with this schedule, median OS is poor, ranging from 10 mo to 15 mo[11]. The role of second-line treatment is debated. Some schedules have been tested, which have also included irinotecan. The use of this drug is suggested due to its efficacy demonstrated in both small-cell lung cancer and in colorectal cancer in several studies[12]. After surgery, to treat local early disease relapse we used second-line chemotherapy with FOLFIRI, as the efficacy of FOLFIRI has already been demonstrated in a French study of 39 patients who failed etoposide and platinum combination[13-15]. After obtaining a PR with systemic treatment, pelvic RT was performed, with a long-term local CR. Two months after the end of second-line chemotherapy, metastatic disease was identified in intra-aortocaval lymph nodes, which showed no response to systemic treatment. The efficacy of radiotherapy in metastatic patients with NECs has been proved in bone and

brain metastasis, but there are no data about its use in other metastatic sites[16,17]. An anecdotal approach with radiosurgery was applied with success in one case of visceral metastasis from rectal MANEC (Mixed Adenoneuroendocrine Carcinoma). Considering the good previous response to radiotherapy, the limited prospects of therapy and the site of oligo-metastasis we decided to treat the patient with another course of RT and obtained a CR.

CONCLUSION

Our case demonstrates how a multimodal approach and continuous follow-up restaging can be successful in obtaining a long-term CR in local and metastatic sites; thus, improving 5-year survival in patients with high grade digestive NEC as confirmed by the last total body CT scan in January 2021 in our patient. Therefore, based on our experience, chemotherapy, RT and surgery should be considered together in the management of rectal NEC even in presence of systemic spread. The treatment for each patient, in the absence of prospective studies and considering the rarity of the disease, need to be accurately tailored.

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REVIEW

Proteoglycans and their functions in esophageal squamous cell carcinoma

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Abstract

Esophageal squamous cell carcinoma (ESCC) is a highly malignant disease that has a poor prognosis. Its high lethality is mainly due to the lack of symptoms at early stages, which culminates in diagnosis at a late stage when the tumor has already metastasized. Unfortunately, the common cancer biomarkers have low sensitivity and specificity in esophageal cancer. Therefore, a better understanding of the molecular mechanisms underlying ESCC progression is needed to identify novel diagnostic markers and therapeutic targets for intervention. The invasion of cancer cells into the surrounding tissue is a crucial step for metastasis. During metastasis, tumor cells can interact with extracellular components and secrete proteolytic enzymes to remodel the surrounding tumor microenvironment. Proteoglycans are one of the major components of extracellular matrix. They are involved in multiple processes of cancer cell invasion and metastasis by interacting with soluble bioactive molecules, surrounding matrix, cell surface receptors, and enzymes. Apart from having diverse functions in tumor cells and their surrounding microenvironment, proteoglycans also have diagnostic and prognostic significance in cancer patients. However, the functional significance and underlying mechanisms of proteoglycans in ESCC are not well understood. This review summarizes the proteoglycans that have been studied in ESCC in order to provide a comprehensive view of the role of proteoglycans in the progression of this cancer type. A long term goal would be to exploit these molecules to provide new strategies for therapeutic intervention.

Key Words: Esophageal squamous cell carcinoma; Proteoglycan; Glycosaminoglycan; Serglycin; Extracellular matrix; Biomarker

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Core Tip: Esophageal squamous cell carcinoma (ESCC) is a highly malignant human cancer because of its early metastasis and late diagnosis. Cancer metastasis involves multiple steps that involve cell-cell and cell-matrix interactions in the tumor microenvironment. Proteoglycans are one of the components of the extracellular matrix that play an important role in cell-matrix interactions. We herein summarize the proteoglycans that have been studied in ESCC to provide a comprehensive view of the role of proteoglycans in ESCC.

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INTRODUCTION

Esophageal cancer is the 7th most common cancer in the world and the sixth highest ranking cancer in terms of mortality rate[1]. The two major subtypes of esophageal cancer are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). People who develop esophageal cancer usually have no specific symptoms at the early stage. The onset of symptoms is often accompanied by difficulty and pain in swallowing (dysphagia), loss of body weight, and heartburn, by which time the tumor is likely already in the advanced stage. Late diagnosis of esophageal cancer is the primary reason for its high lethality. Tobacco use and alcohol consumption are the two main risk factors of ESCC. In addition, genetic susceptibility also plays a role in ESCC. It has been reported that *TP53* has the highest frequency of mutation in ESCC patients[2]. Other frequently mutated genes in ESCC are *RB1*, *CDKN2A*, *PIK3CA*, *CCND1*, *ZNF750*, *NOTCH1*, *NFE2L2*, *FAT1*, and *FAT2*[3-6]. It is widely accepted that tumor progression not only depends on accumulation of genetic alterations but also on changes within the surrounding microenvironment[7]. The contribution of tumor microenvironment and extracellular matrix (ECM) components to cancer development and progression is increasingly being recognized[7-9]. The ECM in the tumor microenvironment consists of proteoglycans, collagens, fibronectin, and laminins[10]. Interaction of these molecules with growth factors, chemokines, cytokines, and matrix metalloproteinases facilitate tumor cell survival, invasion, and metastasis[11,12]. It is a highly dynamic and complex interaction network. In this review, the characteristics of proteoglycans and their functions in ESCC are summarized.

STRUCTURE AND CLASSIFICATION OF PROTEOGLYCANS

Proteoglycans typically consist of polysaccharide chains termed glycosaminoglycan (GAG) and a core protein. The GAGs are covalently attached to the serine residues on the core protein. Proteoglycans differ from glycoproteins in several aspects. For example, the carbohydrate content in proteoglycans is 50%-60%, which is much higher than that in glycoproteins. The GAGs in proteoglycans are linear, negatively-charged long chains, whereas the oligosaccharides in glycoproteins are branched short chains that may or may not be negatively-charged. The GAGs of proteoglycans are composed of repeating disaccharide units of hexuronic acid and hexosamine. They may be modified with sulfate groups at various positions to achieve multiple biological functions[13]. The major categories of GAGs are chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparan sulfate (HS), heparin, and hyaluronan (HA).

CSs consist of repeating disaccharide units of N-acetylgalactosamine (GalNAc) and D-glucuronic acid (GlcA), and can be sulfated at C4 and/or C6 position of the GalNAc unit. The CS chains that contain sulfate group modification at both C4 and C6 positions of the GalNAc unit are named CS-E, whereas those modified exclusively at C4 position of GalNAc are known as CS-A. CS-C contains sulfate group only at C6 position of the GalNAc. CS-D chains have sulfate groups at C6 position of the GalNAc and C2 position of the GlcA. DS chains, also named CS-B, are derived from CS, of which the GlcA residues are epimerized into L-iduronic acid (IdoA). DS chains are

sulfated at C2 position of IdoA and C4/C6 position of the GalNAc. KS chains contain repeating disaccharide units of galactose (Gal) and N-acetylglucosamine (GlcNAc). HS chains consist of GlcA and GlcNAc repeats, while heparins consist of repeating disaccharide units of IdoA and GlcNAc. The sulfate modifications in HS and heparins are in clusters. HA, a linear, protein-free, non-sulfated GAG consists of GlcNAc and GlcA repeating units. The structures of GAG chains are shown in Figure 1.

Proteoglycans can be classified according to their predominant GAG types into CS proteoglycans and HS proteoglycans or according to their cellular and subcellular location, overall gene/protein homology, and the presence of specific protein modules within their respective protein cores into intracellular, cell-surface, pericellular, and extracellular proteoglycans[14]. In this review, the functions of proteoglycans in ESCC will be discussed according to this classification. These proteoglycans are classified and summarized in Table 1.

INTRACELLULAR PROTEOGLYCANS IN ESCC

Serglycin is the only known intracellular proteoglycan. It was first isolated from rat yolk carcinoma cell line L2 (named as pPG1) in 1985[15]. Human *SRGN* (serglycin) was isolated from promyelocytic leukemia HL-60 cells in 1990[16] and from hematopoietic cells in 1992[17]. Its amino acid sequence was later found to be identical to that of human platelet proteoglycan protein, which was isolated and characterized in 1986 [18], and the complete amino acid sequence determined in 1988[19,20].

The human *SRGN* gene is located on chromosome 10 and spans about 16.7 kb with 7% of the gene comprising of exons[16,17,21]. Nicodemus *et al*[16] showed that human *SRGN* gene contains three exons, with exon 1 encoding signal peptide (amino acids 1-27), exon 2 encoding amino acids 28-77, and exon 3 encoding amino acids 77-158, which includes the serine/glycine repeat region (amino acids 94-111). The serine/glycine repeat region is the GAG attachment region that allows the clustering of GAG chains close to the center of the core protein. This structure is unique to serglycin[22].

Serglycin was initially characterized as a hematopoietic proteoglycan[23] and was subsequently found to be present in many other non-hematopoietic cells such as endothelial cells[24], immune cells[25], chondrocytes[26-28], and cancer cells[29,30]. The type and size of the GAG chains of serglycin vary among different cell types and can affect the functions of serglycin. Serglycin synthesized by rat serosal mast cells is mainly modified with heparin or HS, while CS chains are predominant in rat mucosal-like mast cells[31]. HS/CS hybrid GAG chains are found in mouse mastocytoma cells and human erythroleukemia cells[32]. In human umbilical vein endothelial cells, serglycin is modified with CS-GAG chains and has smaller GAG chains than that expressed in platelets[24,33]. In human platelets, the predominant type of GAG chain is CS-4[18].

The enzymes involved in the synthesis of GAG chains have different functions in the particular physiological process of cells. Two enzymes that synthesize CS, namely chondroitin 4-sulfotransferase-1 and GalNAc(4S)-6-O-sulfotansferase, and N-deacetylase/N-sulfotransferase-2, which is essential for heparin synthesis, are positively associated with mast cell maturation, whereas chondroitin 6-sulfotransferase is negatively correlated with mast cell maturation[34]. Serglycin core protein, chondroitin 4-sulfotransferase-1, and GalNAc(4S)-6-O-sulfotansferase are upregulated during mast cell activation and accompanied by downregulation of N-deacetylase/N-sulfotransferase-2[34]. A recent study showed that hyaluronidase-4 can cleave the CS chains of serglycin in human mast cells[35]. These reports suggest that enzymes responsible for GAG synthesis are important in determining the functions of serglycin.

In the past two decades, an ever-increasing number of studies have shown that serglycin plays a significant role in human cancers. It has been reported that serglycin is increased in many human cancers including breast cancer[36-40], multiple myeloma [41-44], acute myeloid leukemia[45], nasopharyngeal carcinoma[46-48], hepatocellular carcinoma (HCC)[49-51], colon cancer[52,53], non-small cell lung cancer[54,55], and glioblastoma[56]. The reported functions of serglycin in promoting cancer progression include regulation of cell adhesion, promoting migration/invasion, inducing angiogenesis, and avoiding immune destruction (Figure 2).

Our recent study showed for the first time that serum serglycin could be a potential non-invasive biomarker with prognostic significance in ESCC[57]. We found that serglycin and its binding partners (including matrix metalloproteinases) could promote ESCC cell invasion, migration, and metastasis. We also identified midkine as a novel GAG-dependent binding partner of serglycin, which contributes to ESCC

Table 1 Classification of proteoglycans[14] with clinicopathological and/or functional significance in esophageal squamous cell carcinoma

Location	Classification	Gene symbol	Eponym	Predominant GAG	Studied in ESCC	Ref.
Intracellular	Secretory granules	*SRGN*	Serglycin	Heparin/CS	Serglycin	Zhu *et al*[57]
Cell-surface	Transmembrane	*SDC*	Syndecan 1-4	HS/CS	Syndecan-1, -2	Mikami *et al*[58], Szumilo *et al*[60] and Huang *et al*[61]
		CSPG4	NG2	CS/DS		
		TGFBR3	Betaglycan	HS/CS		
		PTPRZ1	Phosphacan	CS/DS		
	GPI-anchored	*GPC*	Glypican 1-6	HS	Glypican-1, -3	Hara *et al*[62], Li *et al*[63], Harada *et al*[64] and Song [65]
Pericellular	Basement membrane zone	*HSPG2*	Perlecan	HS/CS		
		AGRN	Agrin	HS		
		COL18A1	Collagen XVIII	HS	Endostatin	Zheng *et al*[71], Zhong *et al* [72] and Chen *et al*[73]
		COL15A1	Collagen XV	CS/HS		
Extracellular	Hyalectans	*ACAN*	Aggrecan	CS/KS		
		VCAN	Versican	CS/DS		
		NCAN	Neurocan	CS/DS		
		BCAN	Brevican	CS/DS		
	SLRPs, Canonical Class I	*DCN*	Decorin	CS/DS	Decorin	Wu *et al*[83], Ji *et al*[84] and Augoff *et al*[85]
		BGN	Biglycan	CS/DS	Biglycan	Zhu *et al*[97]
		ASPN	Asporin			
		ECM2	ECM2			
		ECMX	ECMX			
	SLRPs, Canonical Class II	*FMOD*	Fibromodulin	KS		
		LUM	Lumican	KS	Lumican	Kashyap *et al*[102,103]
		PRELP	PRELP			
		KERA	Keratocan	KS		
		OMD	Osteoadherin	KS		
	SLRPs, Canonical Class III	*EPYC*	Epiphycan	CS/DS		
		OPTC	Opticin			
		OGN	Osteoglycin			
	SLRPs, Non-Canonical Class IV	*CHAD*	Chondroadherin			
		NYX	Nyctalopin			
		TSKU	Tsukushi			
	SLRPs, Non-Canonical Class V	*PODN*	Podocan			
		PODNL1	Podocan-Like 1			
	SPOCK	*SPOCK*	Testican 1-3	HS	Testican-1	Song *et al*[108]

CS: Chondroitin sulfate; DS: Dermatan sulfate; ESCC: Esophageal squamous cell carcinoma; GAG: Glycosaminoglycan; GPI: Glycosylphosphatidylinositol; HS: Heparan sulfate; KS: Keratan sulfate; NG2: Nerve/glial antigen 2; SLRP: Small leucine-rich proteoglycan.

progression by upregulating mitogen-activated protein kinase/extracellular signal-regulated protein kinase signaling and c-Myc expression in an autocrine manner[57].

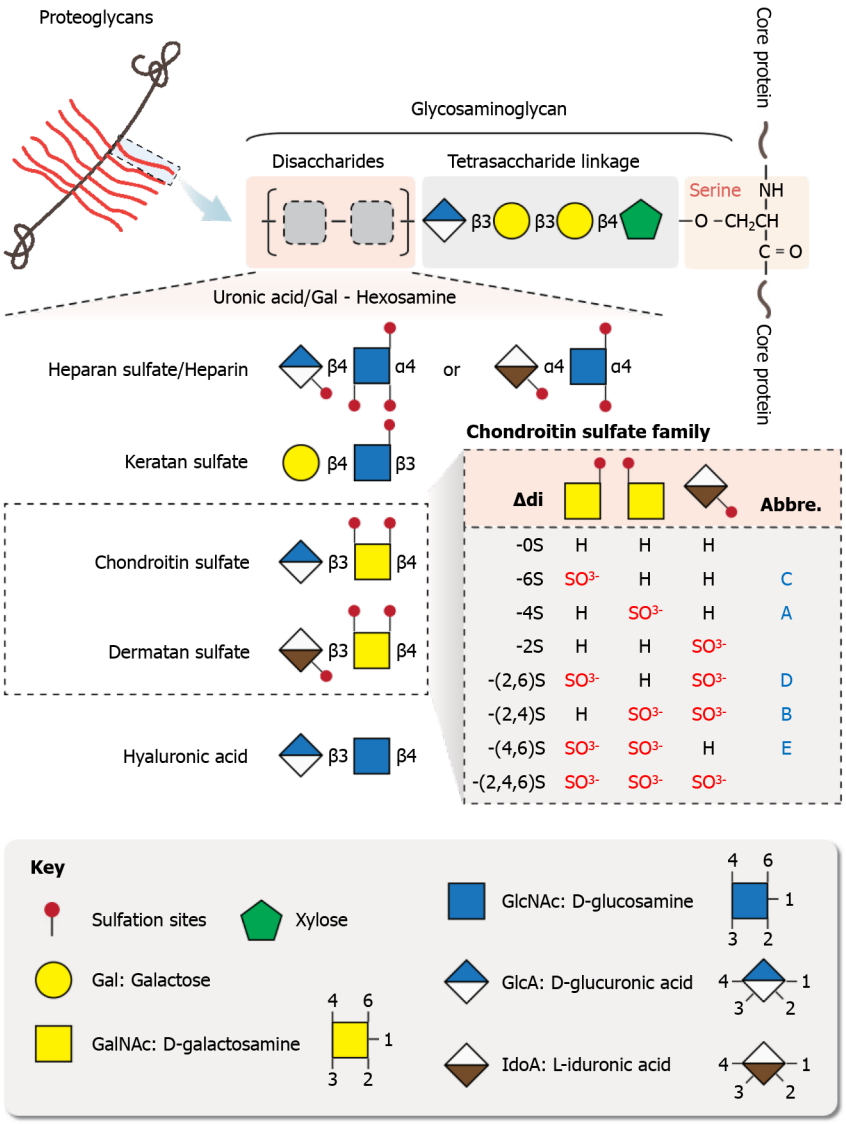


Figure 1 Structure and components of glycosaminoglycan chains of proteoglycans. The disaccharide unit of glycosaminoglycan consists of epimers of uronic acid (GlcA, IdoA) or galactose and hexosamine (GlcNAc, GalNAc). The repeating disaccharide units in different types of glycosaminoglycan are shown with the potential sulfation positions. The different disaccharides types (Δdi) of chondroitin sulfate family have abbreviated names, which are based on their different sulfation positions.

This study also highlighted the significance of CS-GAGs of serglycin in ESCC cell invasion. Strategies that target serglycin, its binding partners, or its GAG chains where most of the protein interactions take place may be a new direction in ESCC therapy.

CELL-SURFACE PROTEOGLYCANS IN ESCC

Syndecan

The syndecan family is a group of transmembrane proteoglycans consisting of syndecan-1, -2, -3, and -4. They generally carry HS-GAG chains. In normal esophageal epithelium, syndecan-1 is strongly expressed in cell membrane, and the expression of syndecan-1 core protein and HS-GAG chains are significantly decreased in T2 and T3 stage specimens compared with lower grade specimens[58]. Although the study by Conejo *et al*[59] found that syndecan-1 messenger RNA level in esophageal malignancies was not significantly different from that in the corresponding normal samples, a histochemical study by Szumilo *et al*[60] showed that well- and moderately-differentiated carcinomas are more frequently syndecan-1-positive compared with poorly differentiated carcinomas. Loss of syndecan-1 was found to be associated with the incidence of lymphatic invasion, and malignant phenotype of ESCC[58]. Notably, the reduction of HS-GAGs on syndecan-1 was more important than that of core protein for

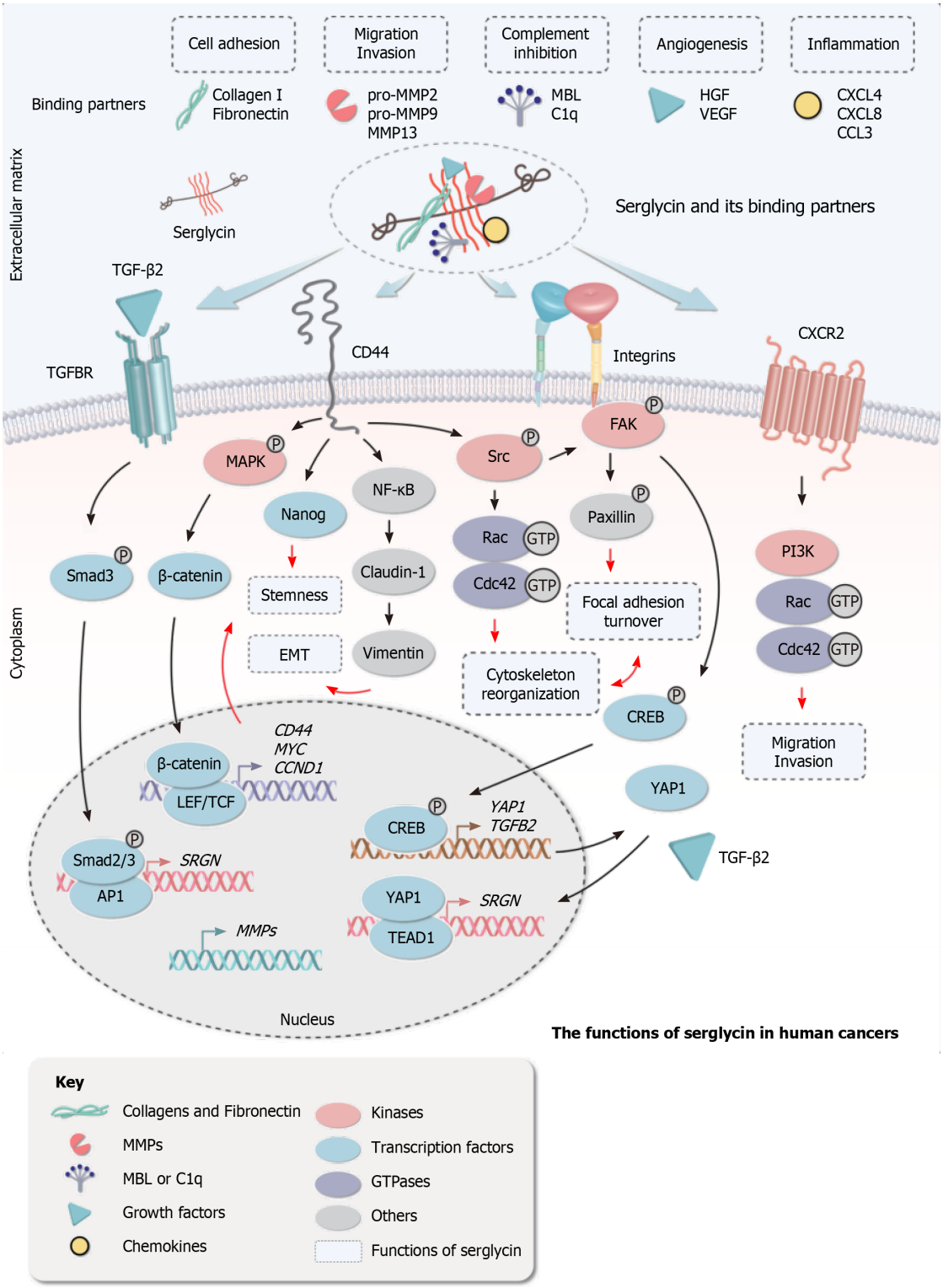


Figure 2 Overview of serglycin functions in human cancers. The various binding partners of serglycin contribute to the multiple functions of serglycin in human cancers. Serglycin, with or without binding partner, can bind to receptors on cancer cells and activate the downstream signaling pathways, including β-catenin, Nanog, phosphatidylinositol-3-kinase (PI3K) and transforming growth factor (TGF) β pathways. MMP: Matrix metalloproteinase; NF-κβ: Nuclear factor-kappa B.

tumor cell invasion; other pathological parameters such as nodal and distant organ metastasis were negatively correlated with HS-GAG expression but not with syndecan-1 core protein expression[58]. Unlike syndecan-1, which acts as a tumor suppressor in ESCC, syndecan-2 is positively correlated with tumor size in ESCC[61]. Multivariate analysis showed that syndecan-2 is an independent prognostic factor for survival rate of ESCC patients after surgery[61].

Glypican

Glypican family includes six members, which are glypican-1 to -6. They also carry HS-GAG chains. Glypican-1 was found to be upregulated in ESCC cell lines compared with normal epithelial cells, and its expression in ESCC tissue is negatively correlated with survival rate of patients[62]. Another study confirmed that ESCC tumor samples have higher expression of glypican-1 than that in para-tumor tissues[63]. Functionally, glypican-1 promotes cell motility and induces epithelial-mesenchymal transition (EMT) in ESCC, possibly through activation of AKT/β-catenin pathway[63]. Knock-down of glypican-1 (*GPC1*) significantly inhibited ESCC cell growth by inhibiting epidermal growth factor receptor pathway and inducing cell apoptosis[64]. Systemic administration of anti-GPC1 antibody significantly inhibited growth of tumor xenografts and tumor angiogenesis[64]. Based on these findings, glypican-1 was described as a promising therapeutic target in ESCC[65].

Unlike glypican-1, glypican-3 did not show significant correlation with histological type, tumor stage, tumor grade, or patient survival in ESCC[66,67], although it was reported to be a diagnostic molecule and a therapeutic target in HCC[68,69].

PERICELLULAR PROTEOGLYCANS

Pericellular proteoglycans include perlecan, agrin, collagen XVIII, and collagen XV (Table 1). The functions of perlecan, agrin, and collagen XV in ESCC have not been elucidated. Endostatin, which is a 20 kDa C-terminal fragment of collagen XVIII, was found to have anti-angiogenic activity[70]. It was later shown to have inhibitory effect on formation of ESCC-related lymphatic vessels[71]. The application of recombinant endostatin protein combined with chemoradiotherapy in ESCC treatment increased the overall survival rate of patients[72]. Recombinant endostatin combined with radiotherapy could significantly inhibit proliferation and migration/invasion of ESCC cells as well as reduce angiogenesis, but there was no effect on cell apoptosis[73].

EXTRACELLULAR PROTEOGLYCANS IN ESCC

Decorin

Decorin, also called PG40, belongs to the small leucine-rich proteoglycan (SLRP) family[74]. The core protein of decorin is about 42 kDa. There is a single GAG chain attached to the N-terminus of the core protein[75]. Proteoglycans belonging to SLRP family contain a region with leucine-rich tandem repeats (LRR). The LRR region is modified by N-glycosylation. N-glycosylation and the O-linked GAG side chains are crucial for the interactions of decorin with other molecules. Studies have shown that the DS-GAGs are essential in fibrillar network formation through bridging collagen fibers[76,77]. The GAG chains and LRR region of SLRPs are both involved in ECM assembly.

Decorin was found to be necessary for appropriate fibrillogenesis due to its ability to bind to collagen[74]. The significance of decorin, as well as other SLRPs, in ECM assembly has been intensively investigated and reviewed[78]. Of note, same classes of SLRPs have the same function of binding to collagen and therefore compete with each other. For example, two other class I SLRPs, namely biglycan and asporin, are able to compensate for the loss of decorin[79]. Asporin can compensate for both decorin and biglycan loss[80]. Lumican, a class II SLRP, can compete with fibromodulin, which belongs to the same class[81,82].

The concentration of plasma decorin in 275 ESCC patients was found to be significantly lower than that in normal controls[83]. The expression of decorin in malignant ESCC tissue samples is also lower compared with normal tissue[84,85]. The study by Ji *et al*[84] showed that decorin expression in ESCC is negatively correlated with histological grade, lymph node metastasis, tumor stage, and clinical stage. Low expression of decorin is associated with poor survival rate and is an independent prognostic marker in patients with ESCC[84]. The tumor suppressive property of decorin, as revealed from studies in other types of cancer such as skin squamous cell carcinoma [86] and breast cancer[87,88], is predominantly due to its ability to trap transforming growth factor β (TGF-β) in the ECM. The binding of decorin to TGF-β prevents the latter from binding to its receptors. Interestingly, this decorin-TGF-β interaction is dependent on decorin-collagen binding[89]. In addition, decorin also acts as a receptor tyrosine kinase inhibitor[90]. It can inhibit the activity of epidermal growth factor

receptor[91], insulin-like growth factor receptor 1, and platelet-derived growth factor receptor α/β[92], thereby suppressing their downstream signaling cascades, and finally inhibiting cancer cell proliferation, migration, and invasion[93]. Systemic administration of recombinant decorin protein can inhibit tumor growth and reduce metastasis of squamous cell carcinoma[86]. Based on these characteristics of decorin, it is regarded as a promising anti-tumor molecule and a potential neoadjuvant therapy for human cancers[94,95].

Biglycan

Biglycan, a SLRP protein coded by the *BGN* gene, is structurally related to decorin but holds two GAG chains rather than one at the N-terminus of the core protein. The molecular weight of biglycan core protein is about 42 kDa. Although biglycan shares similar structure with decorin, the functions of biglycan in human cancers differ from that of decorin. In ESCC, the gene expression of *BGN* is upregulated in tumor samples compared with non-tumor tissues[96,97], although there is no significant association with patient survival[98]. High expression of biglycan in tumor tissue is positively correlated with tumor invasion, lymph node metastasis, and advanced clinical stage [96,97] and is an independent prognostic marker of ESCC[97]. In addition, higher serum biglycan was found in patients with EAC[99], suggesting that biglycan has diagnostic significance in esophageal cancer. Functionally, biglycan has anti-apoptotic effects on mesangial cells and pro-angiogenesis effects on tumor endothelial cells[100], which contribute to cancer cell survival and metastasis. These studies suggest that targeting biglycan may be a novel approach in anti-angiogenic and anti-tumor therapy for ESCC patients[97,100].

Lumican

Lumican is a class II SLRP that has up to three KS-GAG chains. There are contradictory reports on the roles of lumican in human cancers. In pancreatic cancer, patients with high expression of stromal lumican have favorable survival after surgery[101]. However, the gene expression of *LUM* was found to be 7-fold higher in ESCC than in normal epithelia[102,103]. Strong positive lumican immunostaining was found in the stromal and epithelial compartments of ESCC specimens but was almost negative in normal epithelium[103]. The concentration of lumican in plasma was identified as a potential biomarker of ESCC *via* a proteomic screen[104]. Nevertheless, a previous study by our group comparing a highly invasive ESCC subline with its parental cells showed that *LUM* decreased 8-fold in the highly invasive subline and was accompanied by activation of AKT pathway[105]. Li *et al*[101] reported that overexpression of *LUM* suppressed AKT activation in pancreatic cancer cells. These findings infer that lumican might be negatively correlated with AKT activation.

Testican-1

Testican-1, also known as proteoglycan 1, belongs to the SPOCK family and is encoded by the *SPOCK1* gene. It has been reported to be able to induce EMT in gastric cancer [106]. In colorectal cancer, knockdown of *SPOCK1* could significantly reduce cell proliferation and invasion through the inhibition of phosphatidylinositol-3-kinase/AKT pathway[107]. In ESCC, upregulation of *SPOCK1* also induces EMT and promotes cancer cell migration and invasion[108].

CONCLUSION

This article reviews the classification and structure of proteoglycans (Table 1 and Figure 1) and the functions of proteoglycans related to ESCC (Figure 3). As one of the components of the ECM, proteoglycans have been studied in several kinds of human cancers due to their roles in matrix organization and regulation of tumor cell-matrix interactions. Proteoglycans have diagnostic and prognostic significance in ESCC and can modulate ESCC cell migration/invasion and EMT. The secreted serglycin can also activate cell signaling in an autocrine manner (Figure 2). In addition, the significance of GAGs attached to the core protein of proteoglycans (*e.g.*, serglycin and syndecan-1) and the expression level of GAGs regulated by multiple enzymes are increasingly gaining attention. Proteoglycans can enhance or inhibit the activity of soluble factors through interacting with them, and such interactions depend largely on the GAG chains. To date, most studies on proteoglycans in ESCC have focused primarily on their diagnostic and/or prognostic significance. In order to utilize or target these dynamic molecules in designing new strategies for treatment of this cancer, more in-

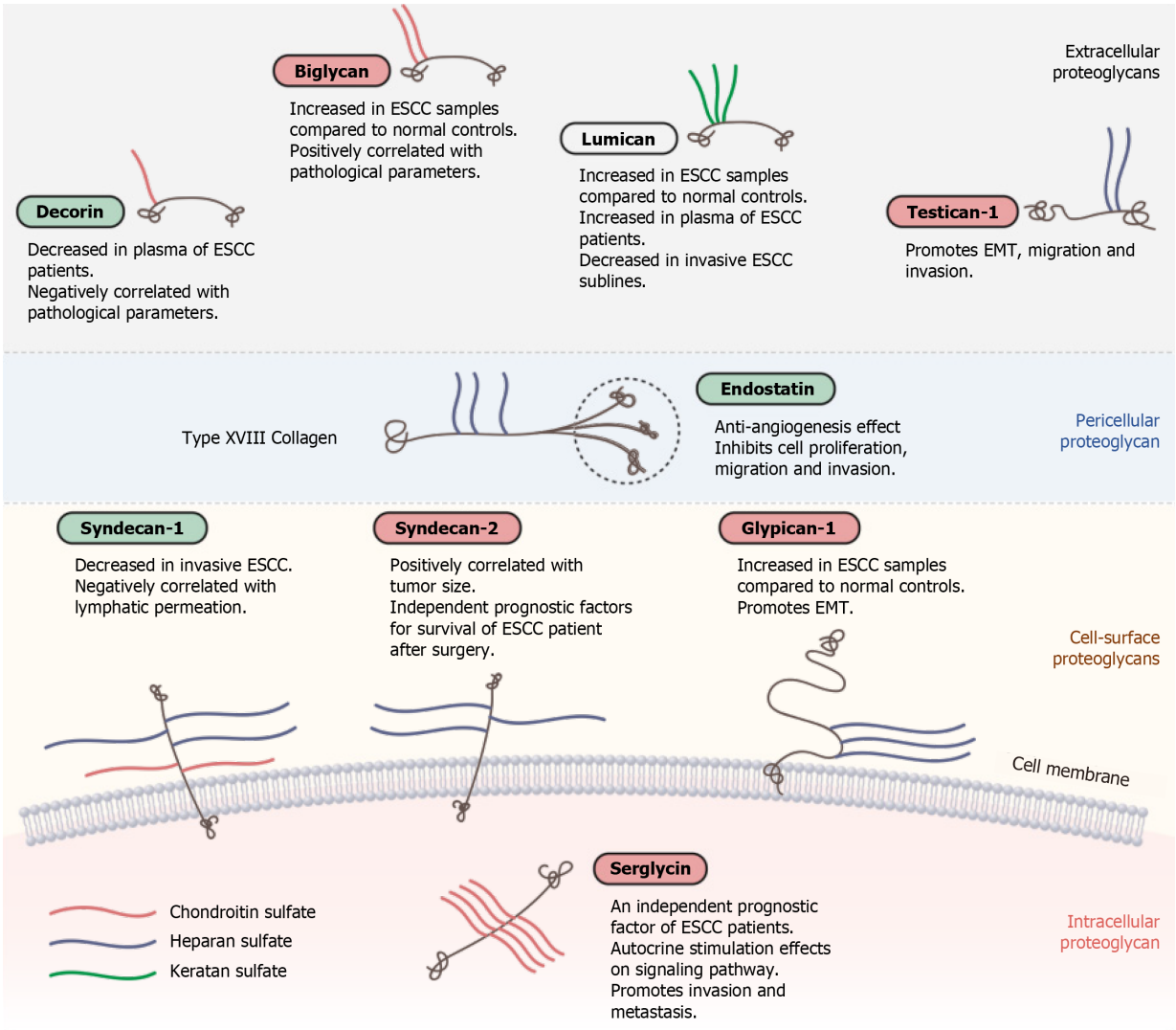


Figure 3 Classification and schematic representation of proteoglycans studied in esophageal squamous cell carcinoma. Proteoglycans are classified as extracellular, pericellular, cell-surface, and intracellular according to their cellular and subcellular localization. The ones that have pro-invasive function in esophageal squamous cell carcinoma (ESCC) are highlighted in red bubbles, and the ones that act as the tumor suppressors are highlighted in green bubbles. Lumican is in the transparent bubble because its function in ESCC is still controversial. EMT: Epithelial-mesenchymal transition.

depth research is needed to decipher the complex roles of proteoglycans in ESCC, especially their interactions with other ECM components, receptors, and soluble factors.

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REVIEW

Therapeutic potential of thymoquinone in combination therapy against cancer and cancer stem cells

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Abstract

The long-term success of standard anticancer monotherapeutic strategies has been hampered by intolerable side effects, resistance to treatment and cancer relapse. These monotherapeutic strategies shrink the tumor bulk but do not effectively eliminate the population of self-renewing cancer stem cells (CSCs) that are normally present within the tumor. These surviving CSCs develop mechanisms of resistance to treatment and refuel the tumor, thus causing cancer relapse. To ensure durable tumor control, research has moved away from adopting the monotreatment paradigm towards developing and using combination therapy. Combining different therapeutic modalities has demonstrated significant therapeutic outcomes by strengthening the anti-tumor potential of monotreatment against cancer and cancer stem cells, mitigating their toxic adverse effects, and ultimately overcoming resistance. Recently, there has been growing interest in combining natural products from different sources or with clinically used chemotherapeutics to further improve treatment efficacy and tolerability. Thymoquinone (TQ), the main bioactive constituent of *Nigella sativa,* has gained great attention in combination therapy research after demonstrating its low toxicity to normal cells and remarkable anticancer efficacy in extensive preclinical studies in addition to its ability to target chemoresistant CSCs. Here, we provide an overview of the therapeutic responses resulting from combining TQ with conventional therapeutic agents such as alkylating agents, antimetabolites and antimicrotubules as well as with topoisomerase inhibitors and non-coding RNA. We also review data on anticancer effects of TQ when combined with ionizing radiation and several natural products such as vitamin D3, melatonin and other compounds derived from Chinese medicinal plants. The focus of this review is on two outcomes of TQ combination therapy, namely eradicating CSCs and treating various types of cancers. In conclusion, the ability of TQ to potentiate the anticancer activity of many chemotherapeutic agents and sensitize cancer cells to

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radiotherapy makes it a promising molecule that could be used in combination therapy to overcome resistance to standard chemotherapeutic agents and reduce their associated toxicities.

Key Words: Thymoquinone; Combination therapy; Cancer cells; Cancer stem cells; Conventional cancer therapy; Natural products

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Core Tip: There has been great interest in integrating thymoquinone (TQ) in combination therapy particularly to target cancer stem cells, which are known to be responsible for resistance to treatment and cancer recurrence. The combination of TQ with standard chemotherapeutics and other natural products has exhibited promising anticancer responses. TQ was also shown to sensitize cancer cells to radiotherapy and help in overcoming major limitations that restrict the potency of chemotherapy, which are chemoresistance and treatment associated toxic effects.

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INTRODUCTION

Cancer incidence and mortality are still growing worldwide despite the monumental efforts and the significant progress made in developing therapeutic strategies and improving detection techniques for combatting this disease. Around 19 million new cases and nearly 10 million deaths are estimated globally in 2020[1]. The conventional therapeutic strategies used to treat cancer are surgery, radiotherapy and chemotherapy, in addition to targeted and hormonal therapy. The effectiveness of these approaches has been found to be limited when used in monotherapy strategies due to cancer resistance, tumor relapse and treatment-induced toxicities[2-6]. Ample evidence has demonstrated that the intratumoral heterogeneity is a prominent contributor to cancer resistance to monotherapy and tumor recurrence[7]. The tumor consists of a heterogeneous population of cells that show distinct genetic, epigenetic, and phenotypic features in addition to different sensitivity to the standard therapeutic modalities[8-11]. A growing body of literature has supported the role of cancer stem cells (CSCs) in generating this intratumoral heterogeneity. These CSCs are characterized by their ability to self- renew and to differentiate into various lineages of cancer cells composing the tumor. They are also resistant to the widely used therapeutics measures [12].

Over the last few decades, there has been increased interest in combining cancer treatments rather than using single therapeutic agents. A monotherapeutic strategy having one mode of action eradicates only one subpopulation of tumor cells. Other subpopulations which are less sensitive can escape the treatment and reform a resistant tumor, thus resulting in cancer relapse and treatment failure. In contrast, combined therapeutic agents act simultaneously on multiple targets and eradicate several subpopulations of tumor cells. This results in improving their therapeutic efficacy, limiting their toxicity by lowering the effective therapeutic dose of each agent, preventing the development of resistance and consequently ensuring an effective eradication of the complex heterogeneous nature of the tumor[13]. Bioactive natural products are attracting considerable attention in cancer therapy because they are less toxic and more available and cost effective when compared to synthetic monotargeted drugs[14]. Natural therapeutics have been found to exert effective antineoplastic activity and to potentiate the anticancer effect of conventional therapeutics against CSCs and cancer cells[15,16]. Around 38% of the anticancer drugs approved during the last 40 years are either natural products *per se*, their derivatives or have a pharmacophore derived from a natural product[17].

Thymoquinone (TQ), the major bioactive compound extracted from *Nigella sativa* essential oil, has shown promising antitumor activity *in vitro* and *in vivo* against a wide range of cancer types[18]. What makes TQ an attractive therapeutic agent is its safe profile. It was found to be non-toxic to several normal cells including normal mouse kidney cells[19], normal human lung fibroblasts[20] and normal human intestinal cells [21]. TQ exerts its antineoplastic effects through several modes of action, and its exact molecular target is not known yet. It inhibits cancer cell proliferation and blocks the cell cycle progression. In addition, TQ induces apoptosis by generating reactive oxygen species (ROS), causing DNA damage, upregulating pro-apoptotic factors, activating caspases and causing poly (ADP-ribose) polymerases (PARP) cleavage, disrupting mitochondrial membrane integrity besides modulating several pathways such as p53, wingless/integrated (Wnt), mitogen-activated protein kinase, signal transducer and activator of transcription 3 (STAT3)[22]. It also interrupts metastasis by downregulating the epithelial to mesenchymal transition transcription factors twist-related protein 1 (TWIST1) and E-Cadherin, and inhibits angiogenesis by suppressing the nuclear factor kappa B (NFkB) pathway[22]. Interestingly, TQ was found to inhibit the proliferation of several chemoresistant cancer cells and induce apoptosis in colon CSCs that are resistant to the conventional chemotherapeutic drug 5-fluorouracil (5-FU)[23,24].

These effective anticancer properties of TQ made it an interesting therapeutic candidate for combination therapy with standard therapeutic agents or other natural products to improve cancer treatment efficacy and safety (Figure 1). Here, we shed light on the combinatorial effects of TQ on the activity of these therapeutic agents used in treating CSCs and cancer cells.

TQ EFFECTS AGAINST CANCER CELLS

TQ in combination with conventional chemotherapeutic agents

The mode of action of each chemotherapeutic agent as well as the cellular and molecular mechanisms of action of the combination treatment are presented in Table 1.

Alkylating agents

Cyclophosphamide[25]: Cyclophosphamide has been used in treating a broad spectrum of cancers including leukemia, lymphoma, breast and ovarian cancers[26]. In a study conducted by Khan *et al*[27], TQ was found to amplify the growth inhibitory effects of low doses of cyclophosphamide in breast cancer cells. This combination upregulated the expression of phosphatase and tensin homolog (PTEN) and downregulated the phosphorylation of its downstream signaling molecule Akt in addition to decreasing the expression of cyclin D1. The PTEN/phosphatidylinositol-3-kinase (PI3K)/Akt pathway is known to be an important tumorigenic pathway responsible for cell cycle progression, survival, and migration of malignant cells[28].

Temozolomide[29]: Temozolomide (TMZ) has been approved by the Food and Drug Administration for the treatment of glioblastoma multiforme[30]. However, the anti-cancer efficacy of TMZ has been limited by cancer resistance[31]. TQ was found to be a potent enhancer of the anti-proliferative and apoptotic activity of TMZ in glioblastoma cells. The modulation of the apoptotic players including ROS generation, disruption of mitochondrial membrane potential, activation of p53, caspases 9 and 3 was more pronounced in combination treatment compared to separate treatments[32]. Moreover, combining TQ and TMZ caused a stronger inhibitory effect on glioblastoma cells migration, invasion and adhesion than each drug alone. This synergistic inhibitory effect was found to be associated with a decrease in the expression and secretion of matrix metalloproteinases MMP-2 and MMP-9[33] known to promote metastatic spread and to contribute to angiogenesis[34]. Interestingly, TQ was found to block TMZ-induced autophagy, which was suggested to be a prosurvival mechanism of cell resistance to TMZ. TQ suppressed TMZ-induced expression of key players in the autophagy pathway beclin-1 and autophagy-related 7[35].

Cisplatin[36]: Cisplatin (CDDP) is one of the most used chemotherapeutic drugs in the treatment of a wide range of cancer types[37]. The primary dose-limiting side effect of CDDP is the dose- dependent nephrotoxicity, which restricts the use of high doses of CDDP to increase its anticancer activity[38]. Numerous studies have demonstrated the anti-neoplastic efficacy of combining TQ with CDDP in different types of cancers as an alternative way to increase CDDP potency. In ovarian cancer, these two agents were

Table 1 Mode of action of the chemotherapeutics agents and cellular and molecular mechanism of action of the combination treatment in preclinical and clinical studies

Chemotherapeutic agent	Mode of action	Patients or animal model or cell lines	Cellular and molecular mechanism of action of the combination treatment	Ref.
Cyclophosphamide	Alkylates guanine base and causes the formation of DNA crosslinks leading to cell death	SKBR-3 and MDA-231 breast cancer cells	Increases the percentage of cells in G1 and sub- G1 phases. Downregulates the phosphorylation of Akt and the expression of cyclin D1 and upregulates PTEN	Emadi *et al*[25], Khan *et al*[27]
Temozolomide	Methylates DNA at specific sites on guanine and adenine bases causing cell demise	U87MG human glioblastoma multiforme cells	Increases the mitochondrial membrane potential disruption, cytochrome c release, ROS generation, DNA fragmentation and Bax/Bcl-2 ratio. Activates p53, caspases 9 and 3 and reduces NO and GSH levels. Reduces the expression and secretion of MMP-2 and MMP-9. Downregulates beclin-1 and ATG-7	Stupp *et al*[29], Khazaei *et al*[32], Pazhouhi *et al*[33], Pazhouhi *et al*[35]
Cisplatin	Interacts with purine bases and forms DNA crosslinks resulting in cell death	ID8-NGL mouse ovarian cancer cells. OVCAR3 and NCI/ADR-RES human ovarian cancer cells. BL/6 mice injected with ID8-NGL cells	Increases the level of Bax, pH2AX (ser139), cleaved caspase 3 and PARP. Decreases the level of PCNA and Ki67	Siddik *et al*[36], Wilson *et al*[39]
		Eca-109 human esophageal cancer cells. BALB/c nude mice inoculated with Eca-109 cells	Decreases the expression of p-STAT3, p-JAK2, Bcl-2, survivin and cyclin D1. Increases the expression of Bax and activates caspases 3, 7 and 9. Induces chromatin condensation and nuclear fragmentation	Hu *et al*[40]
		NCI-H460 non-small lung cancer cells. SCID mice injected with NCI-H460 cancer cells	Reduces the ratio of phosphor-Ser529 NFkB/NFkB	Jafri *et al*[42]
		UMSCC-14C head and neck squamous cancer cells and normal oral epithelial cells	Increases p53 and caspase 9 expression. Decreases Bcl-2 expression	Alaufi *et al*[43]
		SGC-7901 human gastric cancer cells. BALB/c mice implanted with gastric cancer cells	Increases the level of Bax, AIF, cytochrome c, cleaved caspases 9 and 3. Decreases the level of cyclin D1, Bcl-2, procaspases 9 and 3. Inhibits PI3K/Akt signaling pathway and downregulates P-gp by upregulating PTEN	Ma *et al*[44]
5-Fluorouracil	A pyrimidine analogue inhibiting the activity of thymidylate synthase enzyme causing the disruption of DNA synthesis and cell death	BGC-823, SGC-7901, MGC-803 and HGC-27 human gastric cancer cells. BALB/c athymic nude mice inoculated with gastric cancer cells	Increases the release of mitochondrial cytochrome c and the level of Bax, caspases 3 and 9. Decreases the level of Bcl-2 and induces nuclear fragmentation and chromatin condensation	Wilson *et al*[45], Lei *et al*[48]
		Azoxymethane-induced colorectal tumors in Wistar rats	Increases the expression of DKK-1, CDNK-1A, TGF-β1, TGF-βRII, Smad4 and GPx. Decreases the expression of Wnt, β-catenin, NFκB, COX-2, iNOS, VEGF and TBRAS	Kensara *et al*[49]
		HCT116, HT29 and SW620 human colon cancer cellsSW837 rectal cancer cells. Normal human intestinal epithelial cells. CAM tumors derived from HCT116 cells	Downregulates Wnt/β-catenin and PI3K/Akt pathways	Ndreshkjana *et al*[50]
		FADU nasopharyngeal cancer cells	Decreases the level of GSH	Williams *et al*[51]
		MG63 human osteosarcoma cells		Sarman *et al*[52]
Gemcitabine	A deoxycytidine analog preventing chain elongation during DNA	PANC-1 and MIA PaCa-2 human pancreatic cancer cells	Downregulates PKM2 and decreases the expression of procaspase 3 and PARP	Moysan *et al*[53], Pandita *et al*[56]

	causing cell death			
		PANC-1, BxPC-3, and AsPC-1 human pancreatic cancer cell lines. BALB/c nude mice injected with PANC-1 cells	Downregulates Notch1, NICD, Bcl-2, Bcl-xL and XIAP. Inactivates Akt/mTOR/S6 signaling pathway and decreases the phosphorylation and nuclear translocation of p65. Upregulates PTEN, caspases 3 and 9 and Bax and increases cytochrome c release	Mu *et al*[57]
		MCF-7 and T47D human breast cancer cells	Increases pre-G1 cell population	Bashmail *et al*[58]
Paclitaxel	Inhibits microtubules disassembly and induces mitotic arrest	4T1 mouse breast cancer cells. Ehrlich tumor cells. Balb/c mice injected with Ehrlich tumor ascites cells	Increases the level of full length and cleaved caspases 3, 7 and 12 and PARP. Reduces phosphorylated p65 and Akt1. Modulates genes involved in apoptosis, cytokine -cytokine receptor interaction, Fas signaling, p53 signaling and JAK/STAT signaling	Ojima *et al*[59], Şakalar *et al*[63]
		MCF-7 and T47D human breast cancer cells	Increases pre-G1 cell population. Increases the level of cleaved caspase 3 and PARP and the expression of beclin-1 and LC3-II	Bashmail *et al*[64]
		MCF-7 human breast cancer cells		Soni *et al*[65]
Docetaxel	Inhibits microtubules disassembly and induces mitotic arrest	DU-145 human prostate cancer cells	Blocks PI3K/Akt signaling pathway and induces DNA fragmentation	Ojima *et al*[59], Dirican *et al*[69]
		DU-145 and C4-2B human prostate cancer cells	Inhibits PI3K/Akt signaling pathway. Increases the expression of Bax, Bid, caspase 3 and PARP and decreases the expression of Bcl-xL	Singh *et al*[70]
		MCF-7 and MDA-MB-231 human breast cancer cells	Induces DNA damage, cells shrinkage, nuclear fragments, apoptotic bodies and cytoplasmic vacuolation	Alkhatib *et al*[71]
		MCF-7 and MDA-MB-231 human breast cancer cells		Zafar *et al*[72]
		MCF-7 and MDA-MB-231 human breast cancer cells. Balb/c mice healthy or injected with Ehrlich ascites carcinoma cells	Induces nuclear fragmentation and restores the levels of oxidative stress parameters MDA, SOD and GSH. Prevents the alteration of blood cell count and serum biochemical parameters AST, ALT, creatinine and BUN	Zafar *et al*[73]
		MCF-7 breast cancer cells		Odeh *et al*[74]
Cabazitaxel	Inhibits microtubules disassembly and induces mitotic arrest	MCF-7 and MDA-MB-231 human breast cancer cells	Induces DNA fragmentation and increases the sub-G1 population	Ojima *et al*[59], Kommineni *et al*[78]
Doxorubicin	Intercalates DNA, inhibits topoisomerase II, forms free radicals when reduced leading to cell cycle arrest and cell death	Human HTLV-1 positive (HuT-102) and HTLV-1 negative (Jurkat). CD4+ malignant T-cell lines. NOD/SCID mice inoculated with HuT-102 tumor cells	Increases the sub-G1 population and induces ROS production. Disrupts the mitochondrial membrane potential. Downregulates the expression of NFkB and Ki67 and increases the phosphorylation of p53	Meredith *et al*[79], Fatfat *et al*[83]
		HL-60 acute myeloid leukemia cells. Dox resistant HT-29 colon carcinoma cells. MCF-7/TOPO multi-drug resistant breast cancer cells	Induces caspases 3 and 8 activity and ROS generation. Disrupts the mitochondrial membrane potential	Effenberger-Neidnicht *et al*[84]
		BALB/c OlaHsd-foxn1 nude mice injected with MDA-MB-231 breast cancer cells	Induces p38 MAPK phosphorylation and inhibit the expression of XIAP, survivin, Bcl-xL and Bcl-2	Woo *et al*[85]
		SMMC-7721 and HepG2 hepatocarcinoma cells and human normal liver cells HL-7702	Increases caspase 3 and PARP cleavage	Jehan *et al*[86]

		MDA-MB-231 human breast cancer cells. MCF-10A and 3T3 non-neoplastic cells	Induces cell shrinkage, membrane blebbing and apoptotic bodies and disrupts the cell membrane. Increases the Sub-G0 population	Ibiyeye *et al*[87]
		MCF-7 human breast adenocarcinoma and HEPG2 human hepatocellular carcinoma. Albino mice implanted with Heps murine liver cancer cells	Decreases NFkB level and increases that of caspase 3. Increases the level of renal antioxidant enzymes SOD and catalase. Modulates the level of renal oxidative stress biomarkers GSH and MDA. Decreases the level of nephrotoxicity biomarkers BUN and serum creatinine	Zidan *et al*[88]
		Albino transplanted with Ehrlich carcinoma cells	Upregulates p53 and reduces the level of Bcl-2. Decreases the level of cardiac MDA. Decreases the serum level of cardiac markers lactate and creatine	El-Ashmawy *et al*[89]
Topotecan	Inhibits DNA topoisomerase I and causes the formation of irreversible DNA double stranded breaks resulting in cell death. Inhibits hypoxia-inducible factor 1α	U937 acute myelogenous leukemia cells	Increases the sub-G1 population. Increases the expression level of Bax/Bcl-2, p53 and p21 and the cleavage of caspases 3 and 9	Robati *et al*[90], Khalife *et al*[95]
		HT-29 human colon cancer cells	Increases the sub-G1 population. Has no effect on p53, Bax and Bcl-2 expression	Khalife *et al*[96]
Bortezomib	Inhibits the proteasome	U266, H929, KMS, RPMI-8226, RPMI-8226-Dox-6 (doxorubicin-resistant clone), RPMI-8226-LR-5 (a melphalan-resistant clone) human multiple myeloma cells. Balb/c mice implanted with U266 cells	Increases the sub-G1 population and the cleavage of caspase 3 and PARP. Reduces the phosphorylation of NFkB (p65) and the expression of Ki67, VEGF, Bcl-2 and the serum levels of IL-6 and TNF-α	Siveen *et al*[99]
Imatinib	Inhibits tyrosine kinase	HCT116 human colorectal cancer cells	Decreases the expression of ABCB1, ABCG2 and hOCT1. Increases the uptake/efflux ratio of imatinib	Thabet *et al*[103]
Tamoxifen	Competes with estrogen and estradiol for the binding to their receptors and modulates their signaling pathway	MCF-7 and MDA-MB-231 human breast cancer cells		Day *et al*[104], Ganji-Harsini *et al*[106]
		MCF-7, MDA-MB-231, MDA-MB-468, T47D, NIH/3T3 and HaCaT human breast cancer cells. Athymic BALB/c mice injected with MDA-MB-231 cells	Decreases the expression of XIAP and the level of p-Akt, p-Bad, p-MAPK and p-GSK-3β and downregulates the expression of Bcl-xL, Bcl-2 and Ki67. Increases the cleavage of caspase 9 and PARP and induces the expression of Bax, AIF, cytochrome c and p27. Increases the percentage of cells in sub-G1 phase and the fragmentation of DNA	Rajput *et al*[107]
		Breast cancer patients	Increases the tumor tissue catalase, SOD and caspase 3. Decreases the tumor tissue Bcl-2, TGF-β1, MDA, TNF-α and IL-6	Kabel *et al*[108]
Zoledronic acid	Inhibits osteoclast-mediated bone	PC-3 and DU- 145 human prostate cancer cells	Increases DNA fragmentation and activates caspases 3 and 7	Polascik *et al*[109], Dirican *et al*[112]
Arsenic trioxide		Human HTLV-I positive (HuT-102 and C91) and HTLV-I negative (CEM and Jurkat) malignant T-cell lines. NOD SCID mice inoculated with HuT-102 cells	Increases the percentage of cells in Pre-G1 phase, the disruption of the mitochondrial membrane potential and the cleavage of PARP and caspase 3. Upregulates p53, Bax and downregulates XIAP and Bcl- 2	Houssein *et al*[117]

PTEN: Phosphatase and tensin homolog; ROS: Reactive oxygen species; Bax: Bcl-2-associated X protein; NO: Nitric oxide; GSH: Glutathione; MMP: Matrix metalloproteinase; ATG-7: Autophagy-related 7; pH2AX: Phospho-histone 2AX; PCNA: Proliferating cell nuclear antigen; JAK2: Janus kinase 2; STAT3: Signal transducer and activator of transcription 3; NFkB: Nuclear factor kappa B; PI3K: Phosphatidylinositol-3-kinase; AIF: Apoptosis inducing factor; PARP: Poly (ADP-ribose) polymerases; CAM: Chorioallantoic membrane; MAPK: Mitogen-activated protein kinase; COX-2: Cyclooxygenase 2; iNOS:

Inducible nitric oxide synthase; VEGF: Vascular endothelial growth factor; TBRAS: Thiobarbituric acid reactive substances; DKK-1: Dickkopf-related protein-1; CDNK-1A: Cyclin-dependent kinase inhibitor 1A; TGF-β1: Tak transforming growth factor beta 1; TGF-βRII: Transforming growth factor, beta receptor II; GPx: Glutathione peroxidase; GSH: Glutathione; XIAP: X-linked inhibitor of apoptosis protein; mTOR: Mammalian target of rapamycin; PKM2: Pyruvate kinase M2; Bid: BH3 interacting-domain death agonist; AST: Aspartate transaminase; ALT: Alanine transaminase; MDA: Malondialdehyde; SOD: Superoxide dismutase; BUN: Blood urea nitrogen; IL-6: Interleukin 6; TNF-α: Tumor necrosis factor alpha; GSK-3β: Glycogen synthase kinase 3 beta; ABCB1A: ATP-binding cassette subfamily B member 1; ABCG2: ATP-binding cassette subfamily G member 2; hOCT1: Human organic cation transporter 1.

found to synergize to induce apoptosis *in vitro* and in a mouse syngeneic model. The combination was more effective in increasing the levels of Bcl-2-associated X protein (Bax), phospho-histone 2AX on serine 139, cleaved caspase 3 and PARP and in downregulating proliferating cell nuclear antigen compared to CDDP alone[39]. A study conducted by Hu *et al*[40] found that TQ enhanced the apoptotic effect of CDDP in esophageal carcinoma *in vitro* and *in vivo* through downregulating JAK2/STAT3 pathway known to be involved in cancer cell proliferation, survival, angiogenesis and metastasis[41]. Another study showed the synergistic inhibitory effects of the combination of TQ and CDDP on the proliferation of non-small lung cancer cells and on the growth of lung cancer xenografts through the suppression of NFkB[42]. The improvement of CDDP-induced apoptosis by TQ was also demonstrated in oral squamous carcinoma cells. The combination was more potent in upregulating p53 and caspase 9 and downregulating Bcl-2 than CDDP alone[43]. In addition, combining TQ with CDDP resulted in a superior anti-neoplastic activity in gastric cancer *in vitro* and *in vivo* by further upregulating PTEN expression compared to CDDP alone[44].

Antimetabolites

5-FU[45]: 5-FU is the third most frequently used chemotherapeutic drug in the treatment of a variety of solid cancers, but its clinical efficacy is hampered by drug resistance and treatment-associated toxicities[46,47]. It is the second most frequent chemotherapeutic agent that causes cardiotoxicity symptoms[46]. The potential chemomodulatory effects of TQ on 5-FU anticancer activity have been investigated in various cancer types. TQ was reported to chemosensitize gastric cancer cells to 5-FU-induced apoptosis by upregulating Bax, caspases 3 and 9 and downregulating Bcl-2 [48]. Moreover, the combination of TQ with 5-FU synergistically suppressed azoxymethane-induced colorectal tumors initiation and development in rats without causing nephro- and hepato-toxicities. The dual combination enhanced the decrease in the expression level of pro-oncogenic genes [Wnt, β-catenin, NFkB, cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF), and thiobarbituric acid reactive substances] and the increase in the expression level of anti-oncogenic genes [dickkopf-related protein-1 (DKK-1), cyclin-dependent kinase inhibitor 1A (CDNK-1A), transforming growth factor beta 1 (TGF-β1), transforming growth factor, beta receptor II (TGF-βRII), Smad4, and glutathione peroxidase] compared to separate treatments[49]. In another study, Ndreshkjana *et al*[50] linked 5-FU with TQ by esterification to form a new hybrid molecule SARB and tested it on colon cancer cells. Both combination and hybrid treatments enhanced the cytotoxic effects of single agents *in vitro*, while SARB was more effective in suppressing the growth of chorioallantoic membrane xenografts *in vivo*. The cytotoxic effects of 5-FU, TQ and the natural product epigallocatechin-3-gallate in triple and double combinations were evaluated in nasopharyngeal cancer cells. The results revealed that the triple combination had the most potent effect in reducing the total number of cancer cells, and the dual combination of TQ and 5-FU was more effective than the combination of TQ and epigallocatechin-3-gallate[51]. In addition, TQ augmented the apoptotic effects of each of 5-FU and the alkylating agent oxaliplatin in osteosarcoma cells. Interestingly, combining TQ with low doses of each of these drugs was found to produce the same anticancer efficacy as higher doses of these agents[52]. Therefore, this treatment strategy may help in alleviating 5-FU and oxaliplatin undesired adverse effects.

Gemcitabine[53]: Gemcitabine (GCB) has been approved for treating different types of cancer including pancreatic and breast cancers[54]. The therapeutic application of GCB was compromised by several drawbacks including its short half-life in the blood circulation, poor membrane permeability in addition to the development of chemoresistance[55]. TQ and GCB were found to induce synergistic apoptosis in GCB sensitive and resistant pancreatic cancer cells by downregulating pyruvate kinase M2 expression[56]. In another study, pretreatment of pancreatic cancer cells with TQ followed

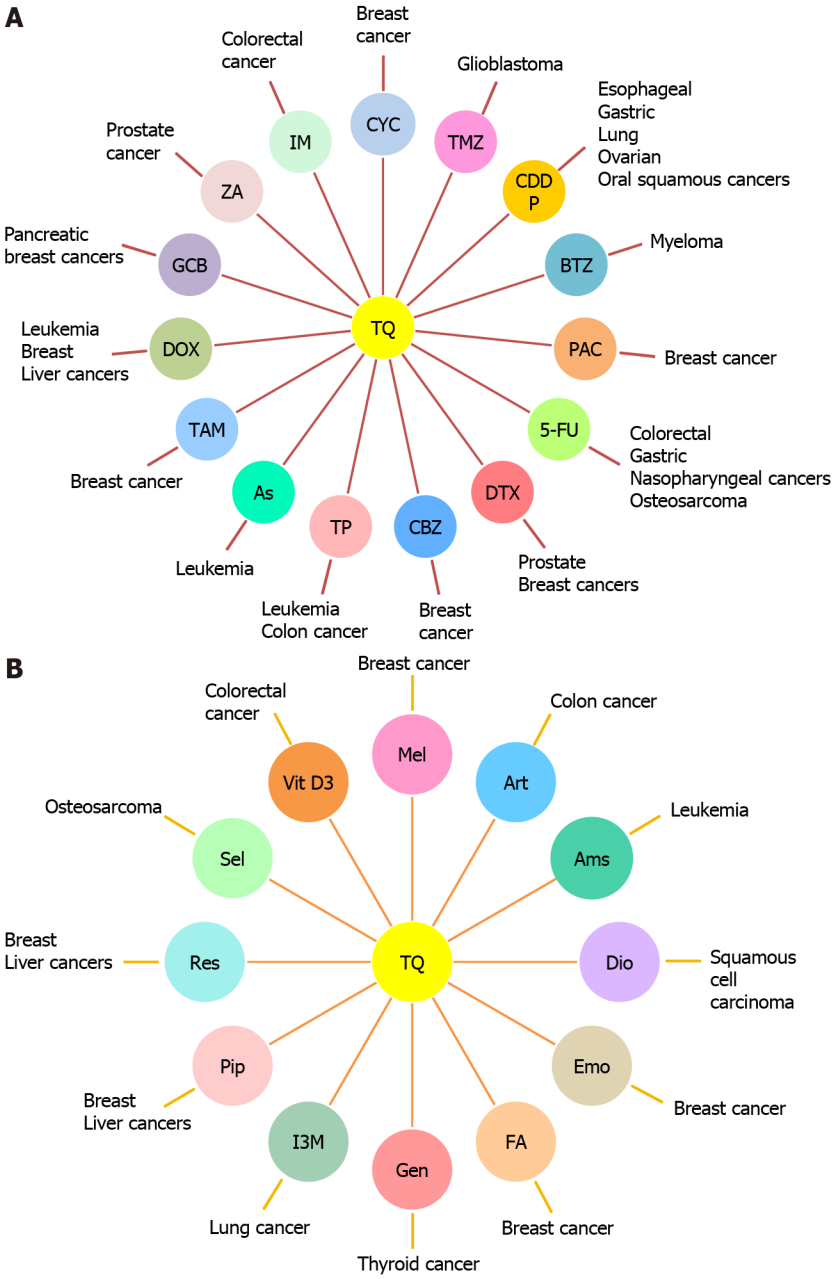


Figure 1 Thymoquinone in combination therapy against different types of cancer. A: Thymoquinone in combination with conventional chemotherapeutic drugs; B: Thymoquinone in combination with natural products. TQ: Thymoquinone; CYC: Cyclophosphamide; TMZ: Temozolomide; CDDP: Cisplatin; BTZ: Bortezomib; 5-FU: 5-Fluorouracil; GCB: Gemcitabine; PAC: Paclitaxel; DTX: Docetaxel; CBZ: Cabazitaxel; TP: Topotecan; DOX: Doxorubicin; ZA: Zoledronic acid; TAM: Tamoxifen; As: Arsenic trioxide; IM: Imatinib; Vit D3: Vitamin D3; Mel: Melatonin; Res: Resveratrol; Pip: Piperine; Ams: Artemisinin; Art: Artesunic acid; Dio: Diosgenin; Gen: Genistein; I3M: Indirubin3monoxime; FA: Ferulic acid; Emo: Emodin; Sel: Selenium.

by low doses of GCB resulted in a synergistic apoptotic and growth inhibitory responses *in vitro* and *in vivo* by downregulating Notch1/PTEN, PI3K/Akt/mammalian target of rapamycin and NFkB mediated signaling pathways[57]. In the context of breast cancer, TQ boosted the apoptotic activity of GCB against T47D cells. While in the apoptosis defective MCF-7 cells, the combination of TQ with GCB induced significant cell death by autophagy[58].

Antimicrotubules

Paclitaxel[59]: Paclitaxel (PAC) is widely used for the treatment of several cancer types including breast, ovary, colorectal and lung cancers[60]. The major challenges that restrict its curative effect are chemoresistance and adverse effects that are mainly caused by the polyethylated castor oil that is usually added to its formulation to increase its solubility[61,62]. Three studies have evaluated the potential of the combinatorial effect of TQ and PAC in breast cancer. TQ-PAC combination produced a

synergistic anticancer activity through the modulation of genes involved in apoptosis, cytokine-cytokine receptor interaction, Fas signaling, p53 signaling and JAK/STAT signaling[63]. In another study, combining TQ with PAC augmented the necrotic and caspase dependent- apoptotic responses in T47D breast cancer cells compared to PAC alone. While in the apoptosis defective MCF-7 cells, both individual and combined treatments induced significant cell death by autophagy[64]. The co-encapsulation of TQ and PAC in polymeric biodegradable poly (lactide-co-glycolide) nanoparticles lowered PAC effective anticancer dose and reduced cancer cell viability more effectively than PAC loaded nanoparticles or its free counterpart[65]. Therefore, this therapeutic approach may help in hijacking the toxicities associated with the clinical use of PAC.

Docetaxel: Docetaxel (DTX) has been approved for the treatment of different type of tumors including prostate cancer and breast cancer[66]. However, low water solubility, treatment related toxicities and drug resistance limit its application in clinical practice[61,67,68]. TQ was found to potentiate the apoptotic activity of DTX in prostate cancer cells by inducing a more prominent suppression of the signaling pathway PI3K/Akt compared to DTX alone[69]. Co-treatment of prostate cancer cells with these two agents resulted in a greater upregulation of Bax, BH3 interacting-domain death agonist (Bid), caspase 3 and PARP and a higher downregulation of Bcl-xL compared to individual treatments[70]. To enhance drug solubility, increase their efficacy and reduce DTX toxicities, multiple nanoparticle drug delivery systems for the co-delivery of TQ and DTX have been developed and evaluated on breast cancer cells. Loading TQ and DTX into a borage nanoemulsion delivery system allowed the lowering of the required effective dose of DTX and enhanced cell death in cancer cells through simultaneous stimulation of apoptosis and autophagy[71]. In another study, co-encapsulating TQ and DTX in low-molecular-weight chitosan coated lipid nanocapsules was found to exhibit stronger cytotoxic and anti-angiogenic responses in cancer cells compared to the free single treatments[72]. The co-delivery of TQ and DTX in Pegylated lipid nanocapsules produced more effective apoptotic and anti-migratory effects in cancer cells in addition to a higher tumor growth inhibition in mice bearing Ehrlich ascites carcinoma compared to free single treatments. Interestingly, these dual drugs loaded lipid nanocapsules prevented the development of DTX-induced hematological, hepato- and nephro- toxicities, an indicator of their protective potential[73]. Moreover, TQ and DTX were co-encapsulated into pegylated liposomes and tested against MCF-7 breast cancer cells. The half maximal inhibitor concentration of each of TQ and DTX co-loaded into liposomes were lower than those of the free individual drugs[74].

Cabazitaxel: Cabazitaxel (CBZ) was approved as the second line therapy for metastatic castration-resistant prostate cancer[75]. However, its low aqueous solubility, poor membrane permeability, and severe side effects like neutropenia and anemia are the challenging drawbacks for successful cancer management[76,77]. Combining TQ with CBZ caused synergistic apoptotic effects in breast cancer cells. To address the drug delivery challenge, TQ and CBZ were co-loaded in lipospheres. The combined drugs loaded lipospheres had enhanced apoptotic effects compared to the drug combination in solution[78].

Cytotoxic antibiotics

Doxorubicin[79]: Doxorubicin (DOX) is a primarily adopted chemotherapeutic agent for treating a wide spectrum of solid and liquid tumors[80]. Despite the robust anticancer activity of DOX, chemoresistance and severe side effects especially cardiotoxicity weakened its potency[81]. Nearly 11% of the patients treated with this agent develop acute cardiotoxicity[82]. Several studies demonstrated the powerful combinatorial effect of TQ on the anticancer efficacy of DOX. Combining TQ with DOX allowed the lowering of DOX dose by up to 2-fold while maintaining its anticancer potential against adult T cell leukemia (ATL). TQ and DOX synergized to induce caspases and ROS mediated apoptosis in human T-lymphotropic virus-1 positive and human T-lymphotropic virus-1 negative CD4+ malignant T cell lines *in vitro* in addition to suppressing the growth of an ATL xenograft in mice[83]. In addition, co-treatment of HL-60 acute myeloid leukemia cells with TQ and DOX induced two consecutives waves of caspase 3 activity in addition to more than 7-fold increase in ROS generation compared to DOX alone[84]. In breast cancer, TQ potentiated the antitumor activity of DOX *in vivo* by inducing apoptosis and inhibiting tumor cell proliferation to a larger extent than separate treatments[85]. Recently, TQ was shown to improve the apoptotic effect of subtoxic doses of DOX in hepatocarcinoma cells by

further increasing the cleavage of caspase 3 and PARP in addition to reducing DOX-induced cytotoxicity to normal liver cells[86]. This synergistic inhibitory effect of TQ and DOX combination was also observed in chemoresistant cancer cells. TQ augmented DOX cell growth inhibitory effect by 2- and 1.2-fold in multi-drug resistant breast cancer cells and in DOX resistant colorectal cancer cells, respectively[84]. To enhance the synergistic effect of these two agents, two nanodrug delivery systems have been developed. Loading TQ and DOX in cockle shell-derived aragonite calcium carbonate nanoparticles (ACNP) showed higher efficacy in inducing apoptosis and reducing migration and invasion in breast cancer cells than the free drugs or the single drug loaded ACNP while being non-toxic to non-neoplastic cells[87]. In addition, incorporating TQ and DOX into F2 gel (poly-N-acetyl glucosamine) nanofibers exhibited superior cellular growth inhibition and apoptosis in breast and liver cancer cells compared to free drugs and single drug loaded nanoparticles. The anticancer potency of this nanodrug co-delivery system was further demonstrated in two *in vivo* cancer models. The dual loading TQ and DOX nanoparticles enhanced tumor suppression *via* apoptosis in mice bearing liver carcinoma by decreasing NFkB level and increasing caspase 3 as well as in mice bearing solid Ehrlich carcinoma by attenuating Bcl-2 level and up-regulating p53. Interestingly, this treatment also reduced the nephro- and cardio-toxicities induced by DOX through the attenuation of the oxidative stress[88,89].

Topoisomerase inhibitor

Topotecan[90]: Topotecan (TP) was approved for the second-line treatment of small cell lung cancer and was recommended to treat platinum resistant ovarian cancer[91,92]. The instability of the chemical structure of TP in aqueous solutions and in the plasma reduces its anticancer efficacy and causes side effects[93,94]. TQ was found to boost the anti-proliferative and apoptotic effects of non-cytotoxic doses of TP in acute myelogenous leukemia and in colon cancer cells. This effect was exerted by upregulation of p53 and Bax, downregulation of Bcl-2, increase in the cleavage of caspases 9 and 3 in leukemia cells and through p53- and Bax/Bcl-2-independent mechanisms in colon cancer cells. In addition, pretreatment of leukemia cells with TQ followed by TP was found to be more effective than the simultaneous application of both therapeutic agents[95,96].

Proteasome inhibitor

Bortezomib: Bortezomib (BTZ) was approved for the treatment of multiple myeloma [97]. It acts by inhibiting NFkB pathway known to be constitutively activated in multiple myeloma due to genetic aberrations in its components[98]. TQ was found to augment the apoptotic activity of BTZ in multiple myeloma cells *in vitro* by enhancing caspase 3 activation and PARP cleavage. In a xenograft multiple myeloma mouse model, TQ potentiated the anti-neoplastic effects of BTZ by further suppressing NFkB and consequently downregulating the proliferative (Ki67), anti-apoptotic (Bcl-2), angiogenic (VEGF) and inflammatory (interleukin-6 and tumor necrosis factor-α) effectors. The authors further showed that TQ reduced the proliferation of BTZ resistant multiple myeloma cells[99].

Tyrosine kinase inhibitor

Imatinib: Imatinib (IM) is a potent tyrosine kinase inhibitor that was approved for treating chronic myeloid leukemia and gastrointestinal stromal tumors[100]. Resistance to IM was reported to develop in cancer patients through several mechanisms including the modulation of the expression of drug efflux and influx transporters[101,102]. In a study conducted by Thabet *et al*[103], TQ was found to improve the anti-proliferative and apoptotic effects of IM in colorectal cancer cells *in vitro*. Interestingly, this was accompanied by a significant decrease in the expression of the drug transporters ATP-binding cassette (ABC) subfamily B member (ABCB) 1, ABCG2 and human organic cation transporter 1 leading to a significant increase in IM uptake /efflux ratio compared to IM alone.

Hormone receptor modulator

Tamoxifen[104]: Tamoxifen (TAM) is one of the first-line therapies for hormone receptor-positive breast cancer patients[105]. A synergistic apoptotic effect was observed by combining TQ and TAM in breast cancer cells *in vitro* regardless of hormone receptor status[106]. Apoptosis was induced through synergistic inhibition of X-linked inhibitor of apoptosis protein (XIAP) resulting in caspase 9 activation and PARP cleavage along with PI3K/Akt pathway inhibition, which caused the downreg-

ulation of Bcl-xL, Bcl-2, and upregulation of Bax, apoptosis inducing factor, cytochrome c and p27. TQ was also found to enhance TAM anti-angiogenic, anti-migratory and anti-invasive effects in breast cancer[107]. In addition, treating breast cancer patients with a combination of TQ and TAM resulted in greater increase in 5-year survival rate and decrease in relapse rate of patients compared to single treatments. At the molecular level, the dual treatment induced a higher increase in tumor tissue antioxidant enzymes (catalase and superoxide dismutase) and increased caspase 3 expression compared to individual treatments. Moreover, the combination of TQ and TAM enhanced the decrease in tumor tissue Bcl-2, TGF-β1, lipid peroxidation product malondialdehyde and pro-inflammatory cytokines tumor necrosis factor-α and interleukin-6 compared to each treatment alone[108].

Biphosphonate

Zoledronic acid: Zoledronic acid is a nitrogen-containing bisphosphonate that inhibits osteoclast-mediated bone resorption. It was approved to prevent and reduce the progression of skeletal complications associated with bone metastasis from solid tumors including prostate cancer[109]. Besides its anti-resorption activity, preclinical and clinical data demonstrated its anti-tumor effects in different types of cancer[110, 111]. TQ intensified the apoptotic activity of zoledronic acid in PC-3 (hormone resistant and chemotherapy sensitive) and DU-145 (hormone and chemotherapy resistant) prostate cancer cell lines through a synergistic increase in DNA fragmentation in both cell lines and a synergistic activation of caspases 3 and 7 in PC-3 cells [112].

Arsenic trioxide

Arsenic trioxide was approved for the treatment of acute promyelocytic leukemia [113]. The combination of arsenic trioxide (As) with interferon alpha (IFN-α) was found to have an effective anti-neoplastic activity in ATL. As and IFN-α synergistically induced apoptosis in ATL leukemia cells *in vitro* and cured murine ATL[114,115]. A phase II trial involving patients with relapsed/refractory adult T-cell leukemia/lymphoma showed that the combination of As and IFN-α exhibited anticancer effects but caused significant toxicity[116]. Combining TQ with As and IFN-α induced synergistic apoptotic activity *in vitro* and *in vivo* and allowed the reduction of the toxic doses of As. TQ alone or TQ/As/IFN-α combination downregulated XIAP and Bcl-2, upregulated Bax and induced cleavage of PARP and caspase 3[117], ultimately leading to enhanced apoptosis.

TQ in combination with ionizing radiation

Radiotherapy is a mainstay therapeutic modality for the treatment of early and advanced solid cancers. Nearly 50% of cancer patients receive radiotherapy during their treatment course[118]. However, its therapeutic potency was found to be compromised by the damage of the surrounding healthy tissue in addition to the development of radioresistance[119]. To overcome these challenges and enhance radiotherapy efficacy, exploring radiosensitizers, molecules that make cancer cells more susceptible to radiations, has attracted great attention[120]. Several studies demonstrated the radiosensitizing role of TQ on cancer cells *in vitro*. TQ augmented the anti-proliferative and apoptotic effects of ionizing radiation and further enriched the sub-G1 population in breast cancer cells[121]. In addition, sensitization with TQ prevented the radiation-induced metastatic progression of breast cancer cells through the restoration of the levels of TGF-β and its downstream effectors in addition to epithelial and mesenchymal markers[122]. In melanoma, TQ enhanced the apoptotic responses of low doses of gamma knife irradiation by further inhibiting the phosphorylation of STAT3, which is known to play a key role in cancer cell proliferation, survival, angiogenesis and metastasis[41]. It also improved the gamma knife irradiation-induced immune response by further attenuating the secretion of tumor-related inflammatory cytokines[123]. The cellular and molecular mechanisms of action of TQ in combination with radiation and other therapeutic agents discussed in this review are presented in Table 2.

TQ in combination with non-coding RNA

Gene therapy is a modern therapeutic approach that demonstrated immense and impressive potential against cancer. It consists of delivering therapeutic genetic materials such as small interfering RNA (siRNA), microRNA, and anti-sense oligonucleotides into cancer cells to restore target gene expression, which is modulated and associated with tumorigenesis[124]. miR-34a is a tumor-suppressive microRNA found

Table 2 Cellular and molecular mechanism of action of the combination treatment in preclinical studies

Therapeutic agent	Animal model or cell line	Cellular and molecular mechanism of action of the combination treatment	Ref.
Radiation	MCF-7 and T47D human breast cancer cells	Increases the percentage of cells in sub-G1 phase	Velho-Pereira *et al*[121]
	MCF-7 and MDA-MB-231 human breast cancer cells	Restores the expression levels of TGF-β and its downstream molecules NFkB, Smad2, Snail and Twist, adhesion molecules E-cadherin and cytokeratin 19, mesenchymal markers integrin αV, MMP-9, and MMP-2	Rajput *et al*[122]
	B16-F10 melanoma cells	Inhibits the phosphorylation of JAK2 and STAT3Increases the expression of caspase 3 and Bax. Reduce the expression of Bcl-2 and survivin and the level of VEGF-A, MCP-1, TGF-β1, RANTES and IL-1β. Induces DNA damage	Hatiboglu *et al*[123]
microRNA-34a	BT-549 metastatic breast cancer cells	Targets and downregulates TWIST1 and ZEB1	Imani *et al*[126]
Akt-siRNA	Akt-overexpressing MCF-7 and T47D. Tamoxifen resistant MCF-7 and T47D breast cancer cells. BALB/c mice injected with MCF-7/TAM cells	Reduces Akt expression and MDM-2 activation. Activates p53, increases the level of Bax and Bim and decreases the level of Bcl-2 and Ki67	Rajput *et al*[127]
Vitamin D3	Azoxymethane-induced colorectal tumors in Wistar rats	Reduces the level of Wnt, β-catenin, NFkB, COX-2, iNOS, VEGF and HSP-90 and increases that of DKK-1, CDNK-1A, TGF-β1, TGF-β/RII and Smad4	Mohamed *et al*[131]
Melatonin	EMT6/P mouse breast cancer cells. Balb/C mice transplanted with EMT6/P cells	Reduces the expression of VEGF and the serum level of AST and ALT. Increases the serum level of IFN-α and decreases that of IL-4	Odeh *et al*[134]
Artemisinin	CCRF-CEM and multidrug-resistant CEM/ADR5000 human leukemia cells. Healthy human foreskin fibroblasts		Fröhlich *et al*[136]
Artesunic acid	HCT116, HT29, Caco-2, DLD-1 colon cancer cells. HCEC nonmalignant colon epithelial cells	Induces ROS generation, DNA damage, PARP and caspase 9 cleavage. Increases the level of γ-H2AX	Fröhlich *et al*[137]
Diosgenin	A431 and Hep2 human squamous cell carcinoma. Swiss albino mice injected with sarcoma 180 cells	Induces DNA fragmentation and cytoskeletal changes. Decreases the expression of CD31 and Ki67	Das *et al*[138]
Emodin	MCF-7, MDA-MB-231, MDA-MB-468 and T47D human breast cancer cells. CAM inoculated with MCF-7 cells	Increases the percentage of cells in sub-G1 phase. Increases ROS generation, cytochrome c release, expression levels of p53, Bax and cleaved caspase 3. Reduces Bcl-2, pFAK and integrinβ1 expression level. Induces nuclear fragmentation, shrinkage, apoptotic body formation, chromatin condensation and membrane blebbing	Bhattacharjee *et al*[140]
Ferulic acid	MDA-MB-231 human breast cancer cells		Al-Mutairi *et al*[143]
Genistein	CALC-62 and ACC448 human thyroid cells derived from anaplastic carcinoma CGTH-W1, ACC360 derived from follicular carcinoma	Reduces the expression level of human telomerase reverse transcriptase, VEGF-A and NFkB. Increases the expression level of PTEN and p21 and activates caspase 3	Ozturk *et al*[145]
Indirubin-3-monoxime	A549 human lung cancer cells. HFL-1 human fetal lung fibroblast. CD1-nude mice injected with A549 cells	Increases the percentage of cells in Sub-G0 phase. Reduces Bcl-2/Bax ratio, TNF-α release and p-Akt (s473), p-mTOR, NFkB/p65, caspase3 and p53 expression level	Dera *et al*[147]
Piperine	EMT6/P mouse mammary cancer cells. Balb/C female mice injected with EMT6/P cancer cells	Reduces VEGF expression. Increases IFN-γ and IL-2 level and caspase 3 activity	Talib *et al*[149]
	HepG2 human hepatocellular cancer cells	Increase ROS generation and decreases GSH and NADPH level	Das *et al*[151]
Resveratrol	HepG2 human hepatocellular cancer cells	Increases caspase 3 activity. Decreases GSH and MDA level	Ismail *et al*[153]
	EMT6/p mouse epithelial breast cancer cells. MCF-7 and T47D human epithelial breast cancer cells kidney epithelial cells. Balb/C mice injected with EMT6/p cancer cells	Induces DNA fragmentation and increases IFN-γ and IL-4 level. Reduces VEGF expression	Alobaedi *et al*[154]

Selenium	MG-63 human osteosarcoma cell line	Increases cellular damage, and decreases the level of alkaline phosphatase and GSH	Barron *et al*[156]

PTEN: Phosphatase and tensin homolog; ROS: Reactive oxygen species; Bax: Bcl-2-associated X protein; GSH: Glutathione; MMP: Matrix metalloproteinases; γ-H2AX: Gamma-histone 2AX; JAK2: Janus kinase 2; STAT3: Signal transducer and activator of transcription 3; NFkB: Nuclear factor kappa B; COX-2: Cyclooxygenase 2; iNOS: Inducible nitric oxide synthase; VEGF: Vascular endothelial growth factor; DKK-1: Dickkopf-related protein-1; CDNK-1A: Cyclin-dependent kinase inhibitor 1A; TGF-β1: Transforming growth factor beta 1; TGF-βRII: Transforming growth factor beta receptor II; GSH: Glutathione; mTOR: Mammalian target of rapamycin; AST: Aspartate transaminase; ALT: Alanine transaminase; MDA: Malondialdehyde; IL: Interleukin; INF: Interferon; TNF-α: Tumor necrosis factor alpha; MCP-1: Monocyte chemoattractant protein-1; RANTES: Regulated on activation normal T cell expressed sequence; TWIST1: Twist-related protein 1; ZEB1: Zinc finger E-box binding homeobox 1; MDM-2: Mouse double minute 2; NADPH: Nicotinamide-adenine dinucleotide phosphate; CAM: Chorioallantoic membrane.

to be downregulated in numerous human cancers including breast cancer[125]. Re-introducing miR-34a in metastatic breast cancer cells targeted and inhibited the expression of epithelial to mesenchymal transition-associated proteins TWIST1, zinc finger E-box binding homeobox 1 and NOTCH1 and suppressed breast cancer cell migration and invasion. Moreover, combining TQ with miR-34a synergistically downregulated TWIST1 and zinc finger E-box binding homeobox 1, suggesting the promising therapeutic potential of this combination against breast cancer metastasis [126]. In another study, multilamellar gold niosomes were developed for the co-delivery of therapeutic Akt-siRNA and TQ to overcome chemotherapeutic resistance induced by Akt overexpression in breast cancer. TQ-siRNA dual loaded niosomes produced stronger anti-proliferative and apoptotic effects in breast cancer *in vitro* and *in vivo* compared to free TQ and TQ loaded neiosomes. The mechanism of the combination treatment involved an effective decrease of the cellular level of Akt which sensitized breast cancer cells to TQ toxicity leading to inhibition of mouse double minute 2 and therefore induction of p53-dependent apoptosis[127].

TQ in combination with natural molecules

Vitamins: Vitamin D3, the active metabolite of vitamin D, was reported to have potent chemopreventive effects against colorectal cancer *in vitro* and *in vivo*[128,129]. In addition, vitamin D supplementation was demonstrated to have clinically positive effects on survival outcomes in patients with colorectal cancer[130]. TQ was found to enhance the chemopreventive effect of vitamin D3 in suppressing the initiation and progression of colon tumors in an azoxymethane-induced rat model of colon cancer. The combination treatment significantly attenuated the number of grown tumors and large aberrant crypts foci. In addition, it decreased the level of pro-oncogenic (Wnt, β-catenin, NFkB, heat shock protein 90 HSP-90) and angiogenic (VEGF, iNOS and COX2) biomarkers and increased the expression of anti-oncogenic (DKK-1, CDNK-1A, TGF-β 1, TGF-β/RII and Smad4) biomarkers compared with individual treatments[131].

Hormones

Melatonin: Melatonin is a natural hormone involved in different biological activities including regulating the circadian rhythm[132]. Ample evidence revealed that melatonin exerts powerful anti-tumor effects through different modes of action including the activation of anticancer immune responses[133]. The combination of TQ with melatonin in breast cancer bearing mice resulted in 60% of cure in treated mice and produced a stronger apoptotic, necrotic and anti-angiogenetic response in addition to a more potent activation of T helper 1 mediated anticancer immune response compared to separate treatments[134].

Plant-derived molecules

Numerous studies have tested the anti-neoplastic efficacy of combining TQ with other plant-derived molecules in different types of cancer. Artemisinin is a sesquiterpene lactone extracted from the Chinese medicinal plant *Artemisia annua*[135]. Fröhlich *et al* [136,137] linked each of Artemisinin and its semisynthetic derivative artesunic acid with TQ *via* covalent bonds and tested the anticancer efficacy of the formed hybrid molecules *in vitro*. They found that the ether-linked artemisinin-TQ hybrid exhibited a potent and selective anti-proliferative activity that was superior to that of the conventional drug DOX against sensitive and multidrug-resistant leukemia cells without being toxic to normal human foreskin fibroblasts[136]. They also found that the ester-linked artesunic acid-TQ hybrid promoted apoptosis mediated by ROS-induced DNA damage in colon cancer cells while being non-toxic to normal colon epithelial cells. The hybrid's effect was found superior to each of the conventional drug 5-FU, the dual and

individual treatments[137]. In another study, Das *et al*[138] demonstrated the synergistic anti-proliferative and apoptotic potential of combining TQ with diosgenin, a natural steroidal saponin isolated from several plants such as *Trigonella foenum-graecum*[139], in squamous cell carcinoma *in vitro* and in a sarcoma 180-induced mouse model. Recently, Bhattacharjee *et al*[140] investigated the combined effect of TQ with emodin, which is a natural anthraquinone obtained from various herbs including *Rheum palmatum*[141]. The results revealed that the dual treatment triggered a synergistic apoptotic response in breast cancer cells and enhanced the reduction of cancer cell migration compared to monotreatment by downregulating two important molecular players, namely focal adhesion kinase and integrin β1. In an *ex ovo* chorioallantoic membrane xenograft model, TQ and emodin were found to suppress the tumor growth and limit the migration of tumor cells to the liver and lung of the chick embryo [140]. Combining low doses of TQ and ferulic acid, obtained from *Ferula asafetida* plant [142], potently inhibited the proliferation of breast cancer cells, while single treatments did not exhibit any inhibitory effects[143]. Moreover, it has been found that the combination of TQ and genistein, a flavonoid found in soybeans[144], resulted in a higher induction of apoptosis in thyroid cancer cells than treatment with either agent alone[145]. In lung cancer, the combination of TQ and indirubin-3-monoxime, a drug derived from the traditional Chinese herbal remedy Danggui Longhui Wan[146], resulted in synergistic apoptotic and anti-migratory effects *in vitro* and synergistic tumor growth suppression *in vivo*[147]. At the molecular level, the dual treatment decreased the phosphorylation of survival-regulatory proteins Akt, mammalian target of rapamycin and NFkB and activated caspase 3 and p53 in animal tumors[147]. Furthermore, combining TQ and piperine, the major alkaloid found in *Piper nigrum L* [148], resulted in a synergistic inhibition of breast cancer *in vitro* and *in vivo*[149]. It induced a high degree of apoptosis and extensive necrosis, inhibited angiogenesis, and stimulated T helper 1 anticancer immune response with no liver and kidney toxicities. Interestingly, TQ was found to play the major role in inducing the caspase-mediated apoptosis[149].

In another study, the encapsulation of TQ and piperine in micro-vehicles made of a natural polymer guar gum extracted from the seeds of *Cymompsis tetraganolobus* plant [150] synergistically reduced the viability of hepatocellular carcinoma cells[151]. This was associated with ROS generation as indicated by an enhanced decrease in the level of intracellular antioxidant glutathione and nicotinamide-adenine dinucleotide phosphate[151]. Two studies assessed the anticancer effectiveness of combining TQ with resveratrol, a stilbene polyphenolic compound extracted from over 70 plants including Polygonum cuspidatum[152]. TQ and resveratrol combination resulted in a greater cytotoxic effect on hepatocellular carcinoma cells compared to single treatments[153]. In addition, TQ and resveratrol synergized to effectively inhibit breast cancer *in vitro* and *in vivo*. The combined drugs induced apoptosis and necrosis, inhibited angiogenesis and stimulated the anticancer immune response without causing liver and kidney toxicities[154]. Co-treating osteosarcoma cells with TQ and selenium, a micronutrient/trace element found abundantly in Astragalus bisulcatus [155], was found to be effective in decreasing cell viability, inducing cellular damage, and attenuating the levels of alkaline phosphatase and glutathione[156].

TQ EFFECTS AGAINST CANCER STEM CELLS

TQ in combination with chemotherapeutics agents

TQ was found to potentiate the effects of each of GCB and PAC in depleting the $CD44^+/CD24^-$ CSCs population within MCF-7 and T47D breast cancer cells[58,64]. In another study, the co-delivery of DOX and TQ in ACNP effectively eradicated breast CSCs enriched from MDA-MB-231 cells cultured in 3D compared to single drug loaded ACNP and drug combinations in solution. The combined drugs loaded ACNP efficiently attenuated the self-renewal potential of breast CSCs as evidenced by the decrease of their mammospheres forming efficiency. This was accompanied by the reduction of breast CSCs markers CD44 and CD24 expression and aldehyde dehydrogenase 1 activity. In addition, the dual drugs loaded ACNP suppressed breast CSCs migration and invasion[157]. In colorectal cancer, combination of TQ and 5-FU as well as their hybrid SARB downregulated two major stem cell regulatory pathways Wnt/β-catenin and PI3K/Akt. In addition, they were found to effectively reduce the self-renewal potential of colorectal CSCs and eradicate $CD133^+$ colorectal CSC population [50].

TQ in combination with natural products

The combined treatment of TQ and emodin improved the elimination of breast CSCs as demonstrated by the enhanced reduction in mammospheres forming efficiency and in $CD44^+/CD24^-$ CSCS population compared to single treatments. Moreover, it down-regulated the stemness promoting transcription factors Oct 4 and SOX2[140].

CONCLUSION

We have emphasized the tremendous potential of TQ in augmenting the anti-neoplastic effects of different therapeutic modalities against a wide range of cancer cells. TQ sensitized cancer cells to radiotherapy and improved outcomes of cancer resistance to conventional chemotherapeutic agents. The use of TQ in combination therapy also lowered the effective doses of standard chemotherapies which helped reduce their associated toxicities while maintaining their therapeutic effectiveness. The combination of TQ with other plant-derived molecules has shown interesting results and merits further investigation to introduce them as potential candidates for treating cancer. Although the studies investigating TQ potency in eliminating CSC in combination therapy are scarce, their results demonstrated great promise. Involving TQ in combination therapy could possibly further eliminate CSCs from tumors and prevent regrowth of neoplasms.

Despite its remarkable anticancer activity, studies reporting TQ anticancer therapeutic potential in clinical settings are still limited due mainly to its hydrophobicity and poor bioavailability. Few studies have supported combined therapies of TQ with nanoparticle formulations to circumvent the drug delivery challenges. These nanoparticles further enhanced the inhibitory effects of the combined agents against cancer or CSC in preclinical studies. Future efforts should be devoted to developing and testing these effective targeted nanoformulations of the combined agents including TQ for potential clinical translation.

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MINIREVIEWS

Mechanisms of acquired resistance of BRCA1/2-driven tumors to platinum compounds and PARP inhibitors

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Abstract

Molecular pathogenesis of tumors arising in *BRCA1/2* germ-line mutation carriers usually includes somatic inactivation of the remaining allele of the involved gene. Consequently, BRCA1/2-driven cancers are sensitive to platinum-based therapy and poly (ADP-ribose) polymerase inhibitors (PARPi). Long-term exposure to these drugs may result in the emergence of secondary *BRCA1/2* mutations, which restore the open-reading frame of the affected allele. This platinum/PARPi cross-resistance mechanism applies both for *BRCA1* and *BRCA2* genes and has been repeatedly validated in various laboratory models and multiple clinical studies. There are some other routes associated with the partial rescue of *BRCA1/2* function or the development of BRCA1/2-independent pathways for genomic maintenance; however, their actual clinical relevance remains to be established. In addition, studies on the short-term neoadjuvant therapy for ovarian cancer revealed that even chemonaive BRCA1-driven tumors contain a small proportion of BRCA1-proficient cells. These pre-existing cells with retained *BRCA1* heterozygosity rapidly repopulate the tumor mass during platinum exposure, but become outcompeted by BRCA1-deficient cells during therapy holidays. Understanding of the platinum/PARPi resistance pathways has led to the development of novel therapeutic approaches, which aim to improve the management of BRCA1/2-related cancers and are currently undergoing preclinical and clinical evaluation.

Key Words: *BRCA1/2* mutations; Platinum-based therapy; Poly (ADP-ribose) polymerase inhibitors; Drug resistance; Secondary mutations; Intratumoral heterogeneity; Neoadjuvant therapy

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Core Tip: BRCA1/2-associated tumors are highly sensitive to platinum compounds and poly (ADP-ribose) polymerase inhibitors; however, they eventually acquire resistance to this type of therapy. Restoration of *BRCA1/2* function *via* the second mutation is the most known mechanism of tumor adaptation to the therapeutic pressure. Some studies demonstrate that even chemonaive BRCA1-driven tumors contain a small fraction of BRCA1-proficient cells suggesting that the loss of the remaining allele of this gene is not the first event in tumor pathogenesis. These pre-existing platinum-resistant cells rapidly repopulate tumor mass during neoadjuvant therapy for ovarian cancer and explain inevitability of the disease relapses after seemingly successful surgical debulking.

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INTRODUCTION

Germ-line mutations in *BRCA1* and *BRCA2* genes are the most well-known cause of hereditary cancer predisposition. *BRCA1/2* pathogenic variants contribute to approximately 5%-10% and 15%-30% breast and ovarian cancer morbidity, respectively[1-6]. In addition, both mentioned genes are involved in the pathogenesis of a subset of stomach cancers, and the inheritance of *BRCA2* inactive alleles is associated with an increased risk of prostate and pancreatic malignancies[7,8]. BRCA1/2-driven tumors tend to have particular clinical characteristics: Being associated with younger age at onset and highly malignant phenotype[9,10]. Breast carcinomas (BCs) occurring in *BRCA1* mutation carriers usually lack the expression of estrogen and progesterone receptors, and BRCA1/2-associated ovarian cancers (OCs) are characterized by serous high-grade histological appearance[10,11].

Breast and ovarian tumors arising in patients with BRCA1/2-associated hereditary cancer syndrome usually develop *via* somatic inactivation of the remaining allele of the involved gene. *BRCA1* and *BRCA2* play a key role in the maintenance of genomic integrity. Consequently, cancers lacking functional *BRCA1* or *BRCA2* proteins are deficient in DNA repair by homologous recombination (HR). Platinum compounds and poly (ADP-ribose) polymerase inhibitors (PARPi) induce massive DNA damage, which requires an HR-mediated repair. BRCA1/2-null cells are deficient for HR and consequently die upon the action of platinum salts or PARPi. This drug sensitivity is tumor-selective, as the normal cells of the patient retain one functional copy of *BRCA1/2* gene and therefore remain capable of coping with the DNA damage[12-14].

As mentioned above, BRCA1/2-related hereditary tumors constitute a significant portion of OCs. In addition, many high-grade serous OCs have other causes of HR deficiency, *e.g.*, somatic biallelic inactivation of *BRCA1/2* genes or the presence of germ-line mutations in other members of DNA repair pathways[1,15]. This explains the high efficacy of platinum-based chemotherapeutic regimens in OC, which were developed empirically before the discovery of *BRCA1/2* genes and constitute a standard-of-care for OC management. As expected, platinum therapy demonstrates increased efficacy in hereditary *vs* sporadic ovarian tumors[2,16]. In contrast to OC, *BRCA1/2* deficiency is characteristic only for a minority of breast tumors; therefore, platinum compounds are not incorporated in the conventional treatment schemes for non-selected BC patients. Several trials demonstrated that platinum salts might outperform other chemotherapeutic agents when applied to BRCA1/2-driven BCs[17-19]. PARPi have been developed specifically for targeting tumors characterized by *BRCA1/2* and/or HR deficiency. There are several PARPi approved for clinical use with slightly varying medical indications[20,21].

Although BRCA1/2-driven tumors have a clear-cut vulnerability, the use of platinum salts or PARPi does not usually result in a cure from metastatic disease. Platinum- and PARPi-exposed cancers eventually manage to escape from the action of BRCA1/2-specific therapy. Multiple preclinical and clinical studies have identified

various BRCA1/2-restoring mechanisms or bypass pathways, which resume resistance to DNA damage in initially HR-deficient tumor cells. Recent investigations also provided evidence for an alternative scenario, where the emergence of the platinum-resistant tumor clone is attributed to a selection of pre-existing BRCA1-proficient cells; these therapy-resistant cells persist in small amounts in chemonaive tumors but are enriched in the residual lesion. This paper provides a brief overview of the mechanisms of acquired platinum and PARP-resistance in BRCA1/2-driven tumors.

RESTORATION *BRCA1/2* BY SECONDARY SOMATIC MUTATIONS

The vast majority of *BRCA1/2* inherited pathogenic alleles are represented by small alterations in the nucleotide sequence, which cause a frameshift and emergence of premature stop-codons. The open reading frame (ORF) can be rescued by a nearby second mutation if it restores an original 3-letter genetic code, or by small deletion, which excises the pathogenic allele and reconstitutes the ORF, or by the true back mutation (Figure 1)[22-24]. A secondary ORF-restoring *BRCA2* mutation was first described in an acute myeloid leukemia cell line obtained from a patient with Fanconi anemia[25]. The discovery of PARPi and the recognition of BRCA1/2-specific action of platinum compounds stimulated intense investigations of the mechanisms of tumor resistance to these drugs. A series of studies revealed that the emergence of secondary *BRCA1/2* mutations is the most reproducible hallmark of the acquisition of a drug-resistant phenotype. Indeed, the reversion mutations have been repeatedly observed in the experiments with cell lines, patient-derived xenografts (PDX) and clinical samples[26,27].

For the time being, the development of secondary ORF-restoring mutations in *BRCA1/2* genes is the only clinically proven mechanism of the tumor adaptation to the therapy, which is relevant both to platinum compounds and PARPi, characteristic both for *BRCA1* and *BRCA2* genes, and has been convincingly validated in patient samples. The true incidence of secondary *BRCA1/2* mutations is difficult to presently define due to various selection biases and technical limitations of available molecular genetic assays: They appear to be found in approximately a quarter of PARPi/platinum-resistant tumors, although some studies provide even higher estimates. Importantly, many reports describe the emergence of multiple distinct *BRCA1/2* ORF-restoring mutations in independent drug-resistant clones obtained from the same patient, thus providing evidence for the functional convergence of tumor adaptation pathways[26,27].

Some data suggest that the genetic reversion is somewhat more characteristic for BRCA2- than for BRCA1-driven tumors. Distinct pathogenic variants of *BRCA1* and *BRCA2* may differ in their ability to be rescued by the second mutation: It is hypothesized that the genetic reversion is more acceptable for non-conservative regions of the above genes, which are more or less dispensable for their function. Indeed, in all cases, except genuine back mutations, the involved region of *BRCA1* and *BRCA2* genes undergoes subtle alterations (*i.e.* the deletion of a few coding nucleotides or the change of the sequence for a few amino acids); therefore, highly conserved parts of these genes may not tolerate this mechanism of genetic adaptation[14,26,27].

In addition to secondary mutations affecting the coding sequence of *BRCA1/2* genes, there are functionally similar events resulting in the production of hypomorphic but still functional protein. For example, loss of exon 11 is compatible with the participation of *BRCA1* in HR; consequently, alternative splicing resulting in the *BRCA1* exon 11 skipping may contribute to the acquired drug resistance[28]. The mutation located in the N-terminal portion of the *BRCA1* gene can be bypassed by the production of a hypomorphic protein, whose translation starts after the frameshift[29]. Another mechanism of the partial rescue of *BRCA1* function involves gene rearrangements, which terminate *BRCA1* translation before the mutation-containing BCRT domain, consequently preventing the proteasomal degradation of *BRCA1*. These truncated versions of *BRCA1* are capable of maintaining HR and mediate PARPi resistance[30]. Upregulation of HSP90 may stabilize some BRCA1-mutant proteins and thus support their function[31]. Amplification of mutated *BRCA2* was shown to compensate for partial loss of *BRCA2* function and rendered PARPi resistance in cell line experiments [32].

Some *BRCA1/2* germ-line pathogenic alleles are represented by so-called large gene rearrangements (LGRs), which may involve deletions of multiple exons. By definition, these tumors cannot be repaired by the second ORF-restoring mutation. One would expect that these tumors are likely to demonstrate a more pronounced and prolonged response to BRCA1/2-specific therapy. *BRCA1/2* LGRs are not specifically considered

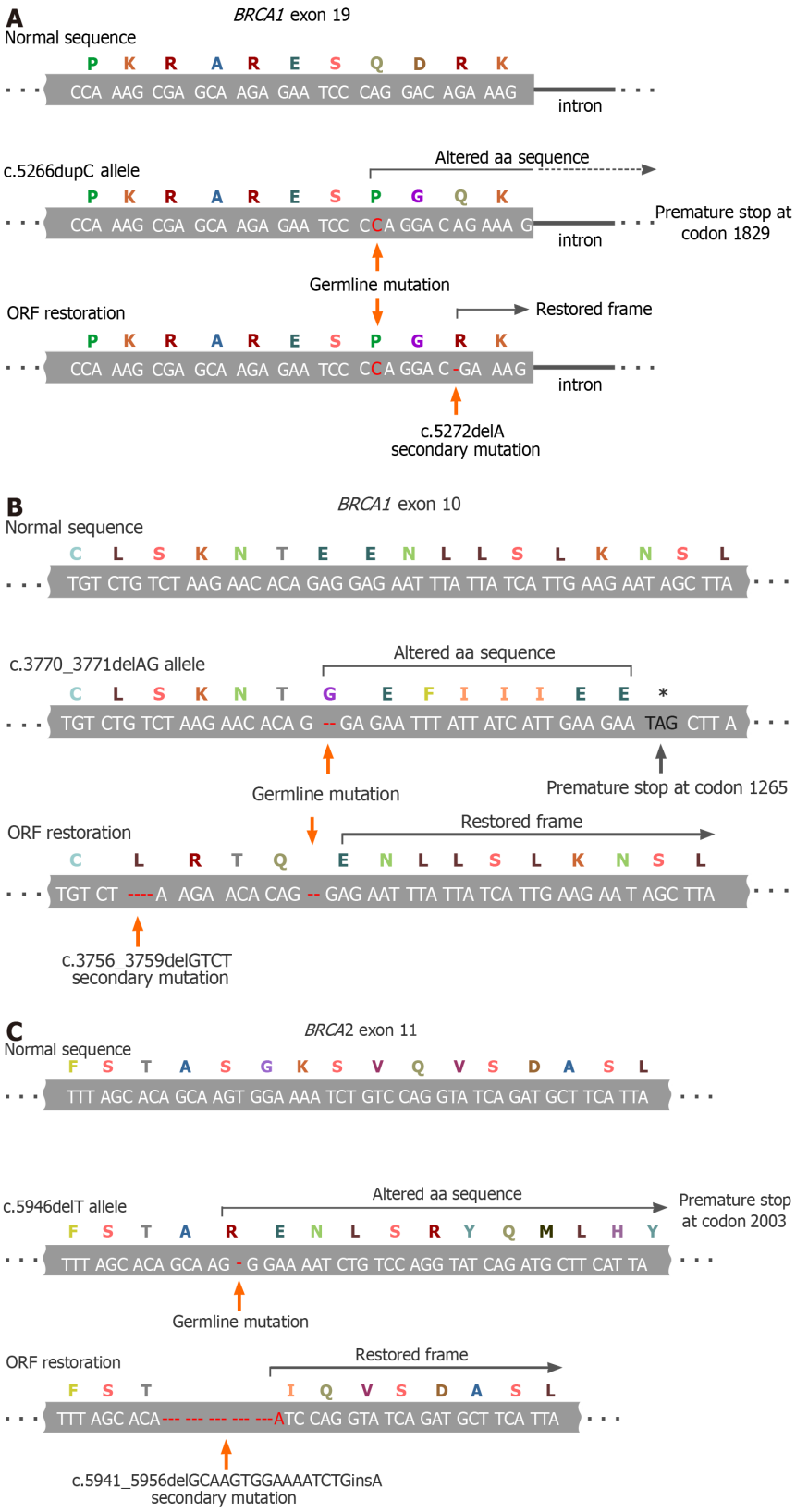


Figure 1 Examples of *BRCA1/2* open-reading frame restoration in BRCA1/2-mutated tumors during systemic therapy. A: Secondary 1-bp deletion occurring downstream to the germline mutation (*BRCA1* c.5266dupC; described in[22]); B: Secondary 4-bp deletion located upstream to the germline mutation (*BRCA1* c.3770_3771delAG; an example from[23]); C: Secondary in-frame deletion/insertion excising the mutation-containing gene fragment (*BRCA2* c.5946delT; an example from[24]). ORF: Open-reading frame.

in the studies on tumor drug sensitivity. However, there are case reports supporting exceptional responsiveness of *BRCA1/2* LGR-associated tumors to PARPi[33].

BYPASS MECHANISMS

There are two key mechanisms of the repair of DNA double-strand breaks (DSB). Accurate correction of DNA sequence can be achieved exclusively by HR. In the absence of functional HR, error-prone non-homologous end-joining (NHEJ) becomes a prevailing mechanism of DSB repair. The choice between HR and NHEJ is mediated by the balance between their regulators, *BRCA1* and *53BP1*. When *BRCA1* is inactivated by mutation, NHEJ-driven DNA repair prevails. This results in the accumulation of multiple DNA lesions and eventual cell death. BRCA1-deficient cells may adapt to the platinum or PARPi pressure by down-regulation of *53BP1*. As a result of consequent NHEJ suppression, tumor cells re-activate HR and eventually become resistant to the drug exposure[31,34,35]. Down-regulation of *53BP1* has been observed in some clinical samples that failed platinum-based or PARPi therapy[31,36,37]. *In vitro* studies revealed several other proteins whose loss also contributes to the switch from NHEJ to HR or to other bypass pathways. Noticeably, the involvement of *53BP1* exemplifies the differences between BRCA1- and BRCA2-mutated tumors, as the loss of *53BP1* or related proteins is relevant only for the treatment escape of BRCA1-deficient cancers[34]. Preclinical studies also identified HR-independent platinum/PARPi resistance mechanisms, which involve stabilization of replication forks[38].

BRCA1/2-NON-RELATED MECHANISMS OF ACQUIRED RESISTANCE TO PLATINUM COMPOUNDS AND PARPi

The above-described mechanisms of acquired therapy resistance are more or less specific for the BRCA1/2-associated action of platinum salts and PARPi. There are also general mechanisms for the adaptation of tumor cells to the therapy, which are indirectly related to the targeted biological pathway and may involve activation of the drug efflux, down-deregulation of apoptosis, preservation of tumor cancer stem cells (CSCs) and epithelial-mesenchymal transition (EMT)[39]. Up-regulation of *ABCB1* (MDR1) transporter has been implicated in multidrug resistance. Therapy-resistant ovarian and breast carcinomas are characterized by gene fusions, which result in increased expression of the *ABCB1* gene[40]. The translational implications of these observations are not immediately clear: Drug transporters are involved in multiple physiological processes and are characterized by significant redundancy, so their targeting may be associated with significant adverse events and insufficient clinical efficacy[41]. There are reports demonstrating the selection of CSCs upon PARPi exposure[42]. The role of EMT in the development of PARPi resistance has been shown in preclinical studies involving BRCA2-deficient cells[43].

SELECTION OF PRE-EXISTING BRCA1-PROFICIENT CELLS DURING PLATINUM-BASED THERAPY

A significant portion of OC patients present with the inoperable disease; therefore, they undergo neoadjuvant (first-line) therapy aimed to reduce the tumor burden and permit surgical excision of the remaining cancer lumps. BRCA1-driven cancers are particularly sensitive to systemic platinum-based treatment; hence, this category of OC is usually amenable to complete surgical debulking. Despite that presumably efficient platinum-based therapy is administered again after the surgery, it apparently cannot eliminate the residual cancer cells, given that almost all OC treated by this scheme eventually relapse[44].

Comparison of tumor specimens obtained before the start of the treatment and after a few weeks of neoadjuvant therapy revealed surprising findings (Figure 2). While chemonaive BRCA1-associated OCs are characterized by somatic loss of heterozygosity (LOH) of the remaining allele, the residual tumors obtained after a few weeks of neoadjuvant therapy often show the retention of the wild-type *BRCA1* copy. This "restoration of heterozygosity" occurs due to the selection of preexisting BRCA1-proficient cells, which persist in small amounts in chemonaive tumors; these isolated tumor cells with retained *BRCA1* function can be visualized by various imaging techniques. Importantly, both primary cancers and residual tumor masses were shown to retain the same mutation in the *TP53* gene. Loss of *BRCA1* in normal cells triggers apoptosis, while cells with inactive *TP53* may survive *BRCA1* deficiency. It appears that *TP53* mutation must be acquired in the very initial stages of the tumor evolution,

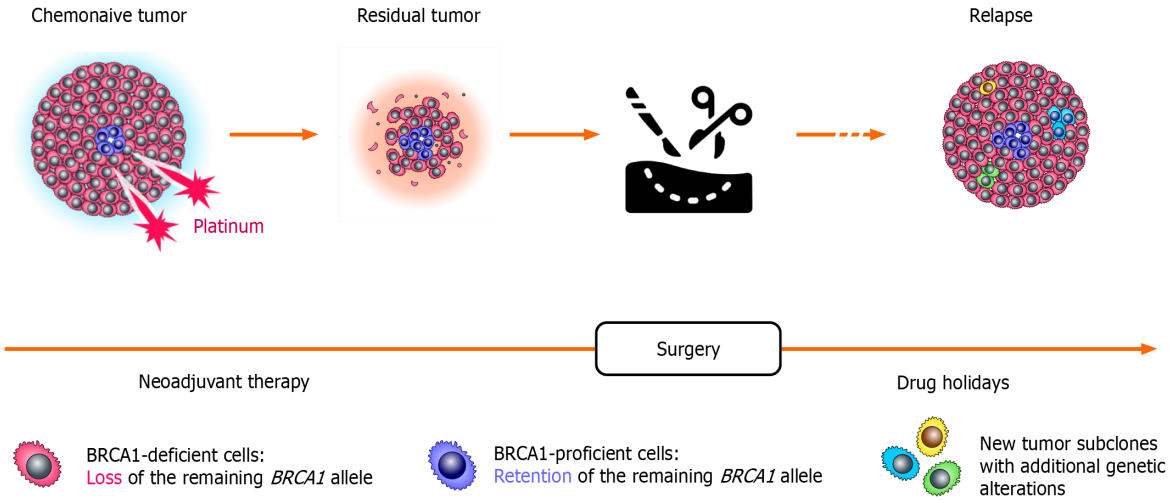


Figure 2 Selection of pre-existing BRCA1-proficient cells during platinum-based therapy. Loss of the remaining *BRCA1* allele is observed in the majority of cells forming the tumor; however, even chemonaive BRCA1-driven ovarian cancers contain a small fraction of transformed cells with retained *BRCA1* heterozygosity. These cells are platinum-resistant and rapidly repopulate tumor mass during neoadjuvant therapy for ovarian cancer. During platinum-free interval, which occurs after the completion of the adjuvant therapy, these BRCA1-proficient cells become outcompeted by cells carrying *BRCA1* LOH. Therefore, ovarian cancer relapses resemble primary tumors with regard to the *BRCA1* status, as they demonstrate again the *BRCA1* deficiency and the sensitivity to platinum compounds.

while *BRCA1* LOH, being a key event in the pathogenesis of BRCA-driven cancers, can emerge and be tolerated only after *TP53* inactivation. The persistence of isolated BRCA1-proficient cells within a gross tumor mass is common for BRCA1-driven cancers, as the "restoration" of *BRCA1* heterozygosity is observed approximately in two-thirds of BRCA1-associated OCs[45].

Intriguingly, the relapse OC tissues obtained from the same patients after therapy holidays show *BRCA1* LOH again, thus providing a mechanistic explanation for the platinum sensitivity of recurrent BRCA1-associated cancers (Figure 2). Exome sequencing revealed that only *TP53* mutation is stably maintained throughout the natural history of BRCA1-driven cancers, while the profiles of somatic point mutations and chromosome number alterations show some variations between chemonaive, post-neoadjuvant and recurrent tumor specimens. Overall, it appears that BRCA1-driven tumors present an ecosystem: While the gross majority of tumor mass is BRCA1-deficient, there are apparently some biological reasons to maintain the persistence of small amounts of BRCA1-proficient cells. In the absence of external hazards, BRCA1-deficient cells clearly outcompete cells with retained *BRCA1* function. However, these cells are sensitive to platinum exposure and perhaps to some other kinds of unfavorable environment, so the maintenance of the reservoir of invulnerable (BRCA1-proficient) cells is important for warranting tumor plasticity. Upon drug pressure, BRCA-proficient cells take advantage and increase their relative fraction in residual tumor mass; however, they again lose the competition after the cessation of the systemic treatment[46]. The above observations fit very well with the concept of tumor "stem cells" as a cause of acquired drug resistance.

The platinum-induced selection of pre-existing BRCA-proficient cells has been demonstrated only for the *BRCA1* gene, while similarly designed studies have not been performed yet for BRCA2-associated tumors. It is not self-explanatory that the same phenomenon is applicable to BRCA2-driven cancers. Indeed, although both *BRCA1* and *BRCA2* proteins are involved in the response to DNA damage, they have essential dissimilarities in their structure and function[14]. Consequently, they demonstrate differences regarding the spectrum of associated tumors, with prostate and pancreatic cancer been strongly linked to *BRCA2* but not to *BRCA1* heterozygosity[7]. Breast carcinomas arising in *BRCA1* germ-line mutation carriers are usually triple-negative with regard to the receptor status (ER, PgR and HER2), while *BRCA2* pathogenic alleles are generally associated with the development of tumors expressing steroid hormone receptors[10,11]. *BRCA1* but not *BRCA2* is essential for taxane-mediated cell death, so the resistance to taxanes is characteristic for BRCA1- but not for BRCA2-deficient cells[12]. The emergence of ORF-restoring secondary mutations in

heavily pretreated tumors appears to be somewhat more common for *BRCA2* than for *BRCA1* gene[26,27]. *BRCA1* deficiency is lethal for normal cells; therefore, the development of cancers in *BRCA1* germ-line mutation carriers always involves mutation-driven inactivation of the *TP53* gene, which results in down-regulation of apoptosis and provides the ground for the survival of BRCA1-null cells. In contrast, *BRCA2* inactivation is compatible with cell viability, so BRCA2-associated tumors often have wild-type *TP53* status[47]. While the persistence of BRCA1-proficient cells in chemo-naive BRCA1-driven tumors is essential for the adaptation of OC to platinum-based therapy, it is unclear how this intratumoral heterogeneity supports the maintenance of tumor mass in "natural" conditions. This intratumoral heterogeneity may not necessarily be characteristic for the cancers arising in *BRCA2* germ-line mutation carriers. Further studies are needed to reveal whether the persistence of isolated HR-proficient "stem" cells is relevant for BRCA2-driven tumors or sporadic OCs with BRCAness phenotype.

CONTROVERSIAL AND UNRESOLVED ISSUES

Platinum compounds and PARPi converge in their mechanisms with regard to targeting HR-deficient cells; however, there are also some differences in their action. For example, platinum salts appear to target tumors with deficient nucleotide excision repair[34]. Consequently, while secondary *BRCA1/2* mutations or other HR-restoring events are likely to result in cross-resistance between platinum and PARPi, other modes of tumor adaptation to the therapy may be more drug-specific. Platinum is commonly used for the treatment of ovarian cancer, and the clinical trials demonstrated that the use of PARPi results in significantly better outcomes in platinum-sensitive *vs* platinum-resistant disease[48,49]. Similarly, the advantage of talazoparib was more pronounced in BRCA1/2-driven breast cancer patients who did not receive prior cisplatin or carboplatin[50]. However, some presumably platinum-resistant ovarian tumors still demonstrate some sensitivity to PARPi[48,49]. On the other hand, PARPi therapy may result, for example, in the emergence of mutations in the PARP1 gene, which alter PARP1 trapping to DNA but are unlikely to affect tumor sensitivity to drugs other than PARPi[51]. It needs to be stressed that in the clinical setting, the platinum sensitivity of ovarian cancer is usually defined not by the actual tumor response to carboplatin or cisplatin but by the time interval exceeding 6 mo since the last platinum exposure. It is not impossible that some tumors may actually restore HR deficiency within a shorter period of time, so their response to PARPi could be explained by conventional PARPi-associated biological mechanisms. While the use of PARPi after chemotherapy has been evaluated in many clinical trials[49], we are unaware of a systematic analysis of chemotherapy response in PARPi-resistant tumors.

BRCA1 and *BRCA2* germ-line mutations are usually viewed as equivalent in all clinical trials involving DNA damaging treatments. Although this approach is generally well justified, some differences between these two genes need to be acknowledged. Preclinical experiments have demonstrated mechanisms for therapy escape that are relevant for BRCA1- but not for BRCA2-driven tumors[34]. The spectrum of associated cancers is somewhat different for these two genes; for example, the analysis of PARPi-resistant prostate malignancies is almost entirely limited to *BRCA2* mutation carriers, as *BRCA1* plays a negligible role in the predisposition to this disease[26].

The regimens of administration of platinum salts and PARPi significantly differ. Cisplatin or carboplatin are usually administered in several cycles, so there are peak drug concentrations and significant intervals between chemotherapy infusions. In contrast to this intermittent drug administration of platinum drugs, PARPi are used at a continuous dose for a prolonged period of time. It is very likely that the mode of drug administration may influence the pathways of tumor adaptation to therapeutic intervention. Furthermore, published clinical experiments included very heterogeneous groups of patients with regard to the duration of prior treatment. It appears that the majority of secondary *BRCA1/2* mutations were detected mainly in heavily pretreated patients, while the initial cycles of chemotherapy rarely resulted in the genetic reversion of the *BRCA1/2* sequence[26,27,46,52].

Cell and animal experiments cannot fully recapitulate the complexity of intratumoral heterogeneity, tumor microenvironment, interplay with the immune system, drug dosing, *etc.*, characteristic for a clinical setting. The investigation of biological material obtained from cancer patients is challenging, particularly when it comes to the analysis of acquired therapy resistance. Tumor re-biopsy, by definition, requires

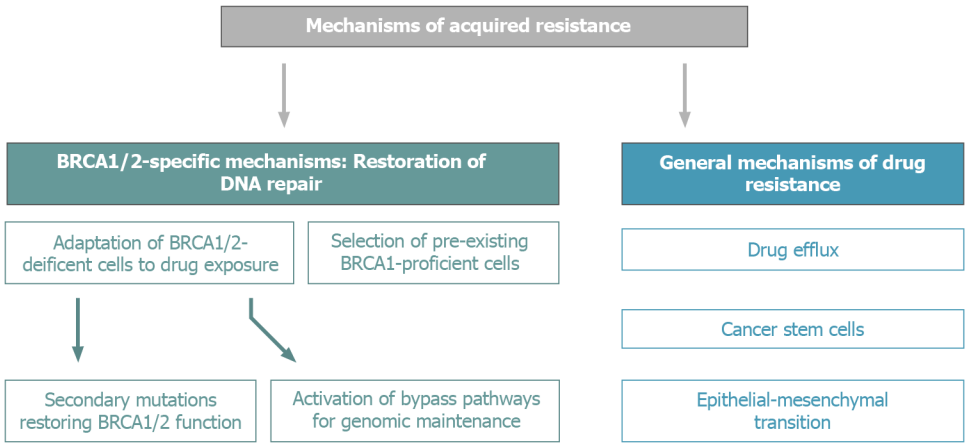


Figure 3 Mechanisms of acquired resistance of BRCA1/2-driven tumors to platinum compounds and PARP inhibitors.

sound clinical and ethical justification; therefore, some studies relied on circulating tumor DNA (ctDNA). Liquid biopsy is capable, in theory, to uncover the entire spectrum of subclonal secondary mutations, although it may underestimate the frequency of back mutations and does not account for the proportion of BRCA1/2-restored cells within a tumor mass. Current technologies for gene sequencing, which are utilized for the detection of secondary mutations, may miss some large deletions of genetic material[26]. It is highly desirable to continue the collection of platinum- and PARPi-resistant tumor samples from cancer patients, to subject these specimens to comprehensive molecular profiling, and to monitor the response of these tumors to subsequent treatment modalities. This effort may identify gene-response correlation and help to guide the clinical management of BRCA1/2-related cancers after the failure of the standard therapy.

CONCLUSION

BRCA1/2-driven tumors have a number of in-built mechanisms of adaptation to conventional schemes of platinum-based therapy and PARPi (Figure 3). Nowadays, an increasing number of OC patients are subjected to long-term PARPi maintenance therapy, which certainly affects the biological and clinical properties of recurrent tumors. It is somewhat surprising that the available medical research literature does not put an emphasis on the potential treatment options for tumors arising on the background of continuous PARPi exposure, despite that multiple lines of preclinical and clinical data suggest the involvement of cross-resistance mechanisms[34].

Genome profiling of drug-resistant tumors obtained from *BRCA1/2* mutation carriers has not identified recurrent actionable molecular lesions[46]. However, despite the restoration of HR proficiency or the emergence of bypass pathways, these tumors continue to contain the genomic scar of BRCAness, *i.e.* the existence of multiple genomic rearrangements. These genetic lesions may underlie an increased antigenicity of BRCA1/2-driven tumors. Interestingly, second mutations, which are the cause of drug resistance, are often associated with the emergence of additional antigenic epitopes[26]. The feasibility of the use of immune therapy against platinum/PARPi-resistant OCs has not been evaluated systematically, although case series support the promise of this option[53].

The best approach would be to implement treatment that would prevent the appearance of drug-resistant clones. There is a number of ongoing trials evaluating the efficacy of combinations of PARPi with other drugs[34,54]. Several studies demonstrated the potentially curative impact of high-dose chemotherapy for *BRCA1/2* mutation carriers; however, the use of this treatment is associated with excessive adverse effects [55]. Neoadjuvant combination of cisplatin and mitomycin C resulted in complete pathological responses, *i.e.* in the elimination of all detectable cancer cells, in some BRCA1/2-driven OCs[44].

There are several recent breakthroughs in the management of BRCA1/2-driven tumors, which resulted in significant improvement of disease outcomes. Continued understanding of the mechanisms of platinum/PARPi resistance inspired the development of a multitude of novel therapeutic approaches, which are likely to

contribute to further advances in cancer treatment. BRCA1/2-associated carcinomas have well-defined vulnerabilities and are characterized by pronounced drug sensitivity. They are similar in this respect to germ-cell tumors and some hematological malignancies, which are generally curable by already available therapeutic tools. There are reasonable chances that cure rates for BRCA1/2-associated malignancies will significantly increase in the near future.

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MINIREVIEWS

Esophagogastric junction adenocarcinoma: Preoperative chemoradiation or perioperative chemotherapy?

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Abstract

Multimodal treatment is currently the standard of care for locally advanced esophagogastric junction (EGJ) adenocarcinoma due to poor results after surgery alone. Neoadjuvant therapy is intended to shrink the tumor and eliminate potential circulating tumor cells. However, which neoadjuvant treatment is best for patients with EGJ tumors remains controversial. We aimed to compare outcomes of preoperative chemoradiation and perioperative chemotherapy for EGJ adenocarcinomas. For this purpose, we performed a thorough review of the literature describing neoadjuvant treatments for EGJ adenocarcinomas or comparing both therapies. Although some studies have shown better locoregional control and higher rates of complete pathologic response after chemoradiation, data suggest that both types of neoadjuvant therapy have similar survival benefits. As current data are heterogeneous and many studies have included significantly different types of patients in their analysis, future studies with better patient selection are still needed to define which neoadjuvant therapy should be chosen. In addition, targeted therapies and immunotherapy have promising results and should be further explored.

Key Words: Esophageal cancer; Esophagogastric junction tumor; Esophageal Adenocarcinoma; Chemotherapy; Chemoradiation; Neoadjuvant therapy

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Core Tip: Surgical treatment only has shown poor results in patients with locally advanced esophagogastric junction tumors. Perioperative chemotherapy and

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neoadjuvant chemoradiation are valid treatment modalities for these patients. This evidence-based review explores the results, advantages, and disadvantages of both approaches. In addition, future directions with potentially effective novel drugs are also discussed.

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INTRODUCTION

Esophagogastric junction (EGJ) adenocarcinoma includes tumors originated from the gastric cardia and the distal esophagus, and is the most common pathological type of esophageal cancer in Western countries[1,2]. The prognosis of this entity is unfavorable in the great majority of patients due to its fast dissemination and advanced disease stages when diagnosed, with an overall 5-year survival of 27%-39%[3,4].

Surgical resection is the gold standard treatment modality for patients without distant disease. The esophagectomy consists of radical resection of the tumor along with the regional lymph nodes[5]. Nevertheless, the poor results after surgical treatment alone have motivated the adoption of neoadjuvant therapies to improve prognosis. Multiple studies have demonstrated that combined preoperative chemoradiotherapy or perioperative chemotherapy plus surgery provide a greater survival benefit than surgery alone[6-9].

Neoadjuvant therapy is intended to shrink the tumor and eliminate potential circulating tumor cells. However, which neoadjuvant treatment is best for patients with EGJ tumors remains controversial. We aimed to compare outcomes of preoperative chemoradiation and perioperative chemotherapy for EGJ adenocarcinomas. For this purpose, we performed a thorough review of the literature describing neoadjuvant treatments for EGJ adenocarcinomas or comparing both therapies.

NEOADJUVANT AND PERIOPERATIVE THERAPIES OVER TIME

Perioperative chemotherapy for EGJ adenocarcinomas has been explored over time. The first milestone was the British Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) trial of 2006, which compared patients with gastric and EGJ adenocarcinomas who underwent 3 cycles of epirubicin, cisplatin, and fluorouracil (ECF) before and after the surgery, *vs* surgery alone. Results showed a significant improvement in R0 resections and an overall survival benefit in patients receiving perioperative chemotherapy[9].

In 2011, a multicenter phase III trial was conducted (ACCORD-07) including patients with resectable adenocarcinomas of the stomach, EGJ, and distal esophagus. They compared surgery alone *vs* perioperative chemotherapy with cisplatin and fluorouracil plus surgery. In patients with resectable adenocarcinomas, perioperative chemotherapy significantly improved overall survival, disease-free survival, and curative resection rates[10].

Shortly after, the ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study (CROSS) Group, published the results of a large study that randomized patients with esophageal or EGJ tumors to surgery alone or preoperative chemoradiotherapy followed by surgery. Patients undergoing preoperative chemoradiotherapy with a 5 wk regimen of carboplatin and paclitaxel followed by concurrent radiotherapy, showed a significant improvement in pathological curative resections and overall survival with acceptable adverse events[7,11].

Finally, the German FLOT4 trial in 2019 compared perioperative ECF *vs* perioperative FLOT (fluorouracil, leucovorin, oxaliplatin, and docetaxel) for gastric and EGJ tumors. This trial was able to demonstrate that patients undergoing FLOT had higher rates of pathological remissions and R0 resections than patients undergoing the MAGIC regimen[12].

Several ongoing trials are currently investigating different neoadjuvant and perioperative therapies in patients with EGJ tumors (Figure 1).

RESULTS OF PREOPERATIVE CHEMORADIOTHERAPY

The CROSS trial included 366 patients [275 (75%) adenocarcinomas, 84 (23%) squamous-cell carcinomas, and 7 (2%) large-cell undifferentiated carcinomas]. The vast majority of patients had distal esophageal cancer, with only 22% of EGJ tumors. Patients were randomly assigned to surgery alone (n = 188) or chemoradiotherapy [intravenous carboplatin (AUC 2 mg/mL per min) and intravenous paclitaxel (50 mg/m^2 of body-surface area) for 23 d] with concurrent radiotherapy (41.4 Gy, given in 23 fractions of 1.8 Gy on 5 d/wk) followed by surgery (n = 178). The chemoradiotherapy group had significantly higher rates of R0 resections (curative resections) than the surgery alone group (92% *vs* 69%; $P < 0.001$). Furthermore, the overall survival was significantly better in the experimental group (49.4 mo *vs* 24 mo), with a 29% of complete pathological response in the neoadjuvant group. In addition, very few adverse events were reported in the chemoradiotherapy-surgery group (6% leukopenia, 5% anorexia, 3% fatigue, and 2% neutropenia)[7].

Long-term follow-up of the CROSS trial confirmed the benefits of neoadjuvant chemoradiotherapy followed by surgery in patients with EGJ and esophageal cancers. Interestingly, in the subgroup analysis by cancer type, patients with squamous cell carcinomas had a greater overall survival benefit than patients with adenocarcinomas [11].

The addition of radiotherapy to the chemotherapy treatment has shown to improved locoregional control by lymph node downstaging and higher rates of complete pathological response (R0 resections). However, this combination might not be highly effective for reducing the risk of distant metastases[2].

RESULTS OF PERIOPERATIVE CHEMOTHERAPY

The British MAGIC trial in 2006 introduced the first perioperative chemotherapy regimen for gastric and EGJ tumors, comparing patients who underwent 3 cycles of ECF before and after the surgery, *vs* patients who underwent surgery alone. The study showed a significant improvement in overall and progression-free survival, as well as higher rates of downsizing of the tumor in the chemotherapy group, with similar complications rates between groups (46% *vs* 45%)[9]. It is worth mentioning that this trial included only 11% of patients with EGJ adenocarcinomas. In addition, few patients were able to complete the full perioperative treatment (91% completed the 3 preoperative cycles, 66% started the 3 postoperative cycles, and only 76% of these patients completed the three cycles), with only 42% of the patients completing the full 6-cycle regimen. Furthermore, no complete pathological response was observed[9].

The French Actions Concertées dans les cancers COloRectaux et Digestifs (ACCORD)-07 trial in 2011 compared patients receiving 2 or 3 cycles of cisplatin and fluorouracil before and after surgery with patients undergoing surgery alone. The authors observed better overall survival (38% *vs* 24%), 5 year disease-free survival (34% *vs* 19%), and higher rates of R0 resections in patients with perioperative chemotherapy[10]. In addition, patients receiving chemotherapy had similar morbidity rates than those undergoing surgery alone. In contrast with the MAGIC-trial, 64% of the patients included in the study had EGJ tumors. However, as well as the MAGIC-trial, one of the main disadvantages was that most patients could not finish the complete regimen due to postoperative morbidity[10].

Based on the results of the MAGIC and ACCORD trials, perioperative chemotherapy was widely embraced for EGJ tumors. In 2019, the German FLOT-4 trial (fluorouracil, leucovorin, oxaliplatin, and docetaxel) analyzed the efficacy and safety of perioperative chemotherapy for locally advanced, resectable gastric and EGJ tumors. In this trial, 716 patients (56% with EGJ tumors) were randomly assigned to perioperative FLOT (n = 356) or ECF (n = 360) plus surgery. An overall survival benefit was observed in the FLOT group (50 mo *vs* 35 mo) and serious adverse events, morbidity, and mortality rates were similar between both groups. These encouraging results have motivated most physicians to adopt FLOT as the standard chemotherapy regimen for patients with EGJ tumors. Remarkably, only 50% and 37% of the patients completed the entire perioperative FLOT or ECF treatment, respectively[12]. Therefore, physicians should be aware that a considerable proportion of patients

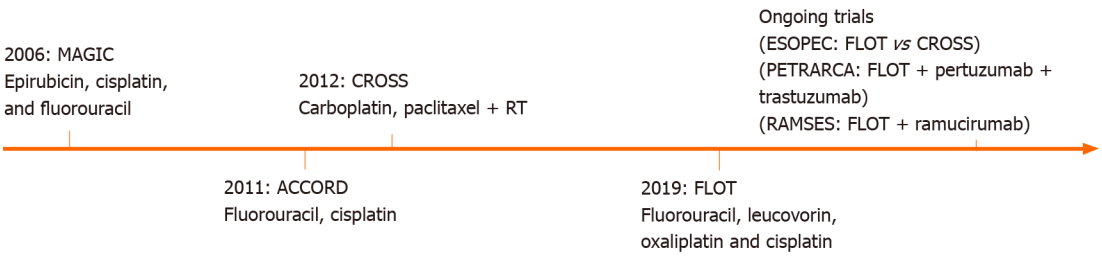


Figure 1 Timeline of neoadjuvant and perioperative therapies in patients with esophagogastric junction cancer. MAGIC: The British Medical Research Council Adjuvant Gastric Infusional Chemotherapy; CROSS: ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study.

might not be able to receive the entire planned systemic treatment. Table 1 describes relevant characteristics of current available neoadjuvant and perioperative therapies.

PERIOPERATIVE CHEMOTHERAPY *VS* PREOPERATIVE CHEMO-RADIATION

To FLOT or to CROSS: that is the question. Regrettably, which is the most effective neoadjuvant therapy for locally advanced EGJ adenocarcinomas remains unclear. In fact, the most important guidelines recommend either perioperative chemotherapy or preoperative chemoradiotherapy for resectable and locally advanced EGJ tumors[13, 14].

Unfortunately, scarce studies have compared both therapies. A recent meta-analysis of 13 randomized controlled trials with almost 5000 patients found no significant differences in overall survival between both regimens (FLOT reached a non-significant HR of 0.88 (95%CI: 0.46-1.62) compared to CROSS for overall survival in random-effects models)[15]. Petrelli *et al*[2] conducted another large systematic review and meta-analysis including 22 studies comparing perioperative chemotherapy and preoperative chemoradiotherapy for GEJ adenocarcinomas, and showed that both therapies had similar overall survival rates. Interestingly, chemoradiotherapy was associated with better locoregional control and higher R0 resection rates but poorer distant metastases control[2].

A propensity score-matched analysis of patients with locally advanced esophageal and EGJ adenocarcinomas compared 40 patients receiving CROSS and 40 receiving FLOT. The study showed that patients undergoing preoperative chemoradiotherapy had higher rates of complete pathological response (97% *vs* 85%; $P = 0.049$) and higher rates of negative lymph node metastases (68% *vs* 40%; $P = 0.014$) than those receiving perioperative chemotherapy. Nevertheless, despite these benefits associated with the CROSS regimen, no difference in overall survival was found between groups[16].

Recently, a study group conducted a propensity score-matched analysis of 3300 patients (1650 for arm) undergoing preoperative chemoradiation *vs* perioperative chemotherapy for resectable lower esophageal and EGJ adenocarcinomas. The authors hypothesized that chemoradiation was superior to chemotherapy. They found that although patients undergoing chemoradiation achieved higher rates of complete pathological response (2.7 times), overall survival was similar in both groups[17]. Similarly, a 2-center retrospective analysis, failed to demonstrate a greater benefit between different neoadjuvant therapies for resectable EGJ adenocarcinomas. They analyzed 85 patients (33 received neoadjuvant/perioperative chemotherapy and 52 neoadjuvant chemoradiotherapy). There was a significantly higher pathological complete response after chemoradiotherapy (30% *vs* 12%; $P = 0.01$). However, these differences did not translate into a different disease-free or overall survival[18].

At our institution, neoadjuvant chemoradiation is mostly used for patients with distal squamous cell carcinoma (Siewert type I). This strategy is based on the subgroup analysis by cancer type of the CROSS trial, which showed that patients with squamous cell carcinomas had greater overall survival benefit than patients with adenocarcinomas.

In patients with EGJ adenocarcinoma, we try to avoid the morbidity of radiation and we usually offer perioperative chemotherapy based on the multiple trials showing good outcomes with this approach (MAGIC, ACCORD, and FLOT). Currently, we offer FLOT regimen due to the recent results of the FLOT trial. Radiation is usually added in patients with extensive loco-regional involvement (*i.e.* bulky tumors).

Table 1 Relevant characteristics of current available neoadjuvant and perioperative therapies for esophagogastric junction tumors

Study	Year	Number of patients	Included patients	Groups	EGJ tumors	Outcomes
MAGIC	2006	503	Gastric, lower esophagus, and EGJ tumors	ECF + Surgery *vs* Surgery alone	11%	Perioperative chemotherapy improves overall survival
ACCORD	2011	224	Gastric, lower esophagus and EGJ tumors	CF + Surgery *vs* Surgery alone	64%	Perioperative chemotherapy improves overall survival, disease-free survival and resecability
CROSS	2012	366	Esophageal and EGJ tumors	Chemoradiation + Surgery *vs* Surgery alone	22%	Chemoradiotherapy improves overall survival
FLOT	2019	716	Gastric and EGJ tumors	FLOT *vs* ECF	56%	FLOT improves overall survival

EGJ: Esophagogastric junction; MAGIC: the British Medical Research Council Adjuvant Gastric Infusional Chemotherapy; ECF: Epirubicin, cisplatin, and fluorouracil; CROSS: ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study.

Overall, further studies are needed to clarify which is the best neoadjuvant treatment for EGJ tumors. Each neoadjuvant modality has advantages and disadvantages that should be considered in a case-by-case basis (Table 2).

FUTURE DIRECTIONS

As no study could demonstrate greater benefit between chemoradiotherapy or perioperative chemotherapy for resectable EGJ adenocarcinomas, efforts to elucidate the best multimodal treatment are still needed.

The ongoing multicenter randomized controlled phase III ESOPEC-trial compares neoadjuvant CROSS *vs* FLOT in patients with resectable and potentially curative esophageal adenocarcinoma. The authors hypothesized that the FLOT regimen might improve overall survival and distant metastases disease control. The results of this trial will hopefully help to decide the most suitable neoadjuvant therapy for patients with EGJ adenocarcinoma[4].

Targeted therapies are designed to inhibit specific molecules overexpressed in patients' tumors and are also currently explored for the treatment of esophageal cancer. The human epidermal growth factor receptor 2 (HER2) is involved in diverse cellular functions such as cell growth, differentiation, and survival. Trastuzumab is a monoclonal antibody targeting the extracellular domain of HER2. The trastuzumab for gastric cancer (TOGA) trial evaluated patients with advanced gastroesophageal adenocarcinoma with overexpression of HER2, and found that the addition of trastuzumab to standard chemotherapy was associated with improved overall survival [19]. A recent trial, however, did not show a survival advantage with the addition of trastuzumab to neoadjuvant chemoradiation in patients with HER2 overexpressing esophageal adenocarcinoma[20]. Pertuzumab is another monoclonal antibody targeting HER2. The PETRARCA trial is currently evaluating the outcomes of perioperative trastuzumab and pertuzumab in combination with FLOT *vs* FLOT alone for patients with HER2-positive resectable esophagogastric adenocarcinoma[21].

The vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) regulate angiogenesis and play a key role in tumor growth. Bevacizumab (monoclonal antibody against VEGF-A), ramucirumab (monoclonal antibody against VEGFR-2), and apatinib (molecule inhibitor selective for VEGF-2) are some of the drugs under investigation[22-25].

The advent of immunotherapy has also brought hope for the treatment of esophageal cancer. Immunotherapy utilizes monoclonal antibodies directed against immune checkpoint proteins such as program death 1 (PD-1) receptor, programmed death ligand 1 (PD-L1) or cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). Recent studies have demonstrated survival advantages with monoclonal antibodies targeting PD-1/PD-L1 (*e.g.*, pembrolizumab, nivolumab) in patients with advanced gastric esophageal cancer[26].

Although targeted therapeutics and immunotherapies are indeed promising, further studies are needed to define the safety and efficacy of these drugs.

Table 2 Potential advantages (+) and disadvantages (-) of preoperative chemoradiation and perioperative chemotherapy

Preoperative chemoradiotherapy	Perioperative chemotherapy
+ Better loco-regional control	+ Better systemic control
+ High rates of complete pathologic response	+ No adverse events from radiotherapy
- Poorer response in adenocarcinoma (increased radiation sensitivity in squamous cell carcinoma)	- Poorer loco-regional control
- Radiation-induced changes in surgical field	- Many patients are not able to complete the postoperative regimen

CONCLUSION

Although some studies have shown better locoregional control and higher rates of complete pathologic response after chemoradiation as compared to perioperative chemotherapy, current data suggest that both types of neoadjuvant therapy have similar survival benefits. Future studies comparing both treatment modalities and with better patient selection are still needed to define which neoadjuvant therapy should be chosen. Targeted therapeutics and immunotherapies have promising results and might also be part of the treatment armamentarium in the future.

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MINIREVIEWS

BRCA mutations and gastrointestinal cancers: When to expect the unexpected?

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Abstract

BRCA1/2 pathogenic variants are widely known as major risk factors mainly for breast and ovarian cancer, while their role in gastrointestinal (GI) malignancies such as colorectal cancer (CRC), gastric cancer and oesophageal cancer (OeC) is still not well established. The main objective of this review is to summarise the available evidence on this matter. The studies included in the review were selected from PubMed/GoogleScholar/ScienceDirect databases to identify published articles where BRCA1/2 pathogenic variants were assessed either as a risk factor or a prognostic/predictive factor in these malignancies. Our review suggests that BRCA1/2 might have a role as a risk factor for colorectal, gastric and OeC, albeit with differences among these diseases: In particular BRCA1 seems to be much more frequently mutated in CRC whereas BRCA2 appears to be much more closely associated with gastric and OeC. Early-onset cancer seems to be also associated with BRCA1/2 mutations and a few studies suggest a positive prognostic role of these mutations. The assessment of a potentially predictive role of these mutations is hampered by the fact that most patients with these diseases have been treated with platinum compounds, where it is expected that a higher probability of response should be seen. A few clinical trials focused on poly (ADP-ribose) polymerase inhibitors use in GI cancers are currently ongoing.

Key Words: BRCA; Colorectal cancer; Gastric cancer; Esophageal cancer; Prognosis; Poly (ADP-ribose) polymerase inhibitors

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Core Tip: BRCA1/2 pathogenic mutations, other than increasing the risk of breast and ovarian cancer, may increase the risk of developing other types of cancers, such as prostate and pancreatic cancers. Our review highlights that BRCA mutations could also have a predisposing role to gastrointestinal tumours, in particular gastric, esophageal and colorectal cancer, thus playing an important role in terms of surveillance procedures and therapeutic options.

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INTRODUCTION

BRCA mutations are defined as pathogenic variants in either the *BRCA1* or *BRCA2* gene: These two tumour suppressor genes are involved in different crucial pathways including DNA repair, cell proliferation control and apoptosis. In particular, *BRCA1* and *BRCA2* genes are mainly involved in the homologous recombination (HR) process, responsible for maintenance of genome integrity through an error-free repair pathway for DNA double-strand breaks in response to DNA damage[1,2]. Loss-of-function mutations in BRCA1/2 may lead to accumulation of DNA double-strand breaks and result in genomic instability and tumour development.

BRCA1 and BRCA2-associated hereditary breast and ovarian cancer syndrome is characterised by an increased risk of breast cancer and ovarian cancer (including fallopian tube and primary peritoneal tumours), up to 84% for breast cancer and 40% for ovarian cancer[3-5]. Moreover, higher risk of male breast cancer has been reported especially in BRCA2 carriers[6-9].

In BRCA2 pathogenic variant carriers, familial cancer types different from breast and ovarian, such as prostate, pancreatic cancer, and melanoma, have also been described[10-14].

In addition to that, case-control and family-based studies seem to suggest that BRCA1/2 pathogenic germline carriers might be at increased risk of other malignancies, although this seems less likely[6,15,16]. It is unclear whether BRCA1 and BRCA2 mutations might determine increased risk of tumour types that are already quite common in the general population, such as gastrointestinal (GI) cancers: This might be due to the fact that most data on the subject is derived from cross-sectional studies of family histories of women with BRCA pathogenic mutations.

These studies might be susceptible to selection bias; furthermore, misclassification can be present, due to the fact that GI cancer diagnoses were based only on information provided by a family member and this approach may not always be reliable.

Moreover, prospective evidence estimating GI cancer incidence in BRCA1/2 pathogenic variants carriers are still rare[17]. Predicting GI cancer incidence rates among BRCA carriers might convey meaningful implications both for genetic counsellors and tested individuals, as it would aid in the development of appropriate screening policies and risk reduction procedures. Indeed, at the moment, specific guidelines or recommendations regarding the need for gastric and bowel screening procedures for carriers of BRCA1/2 mutations are lacking.

Furthermore, BRCA1 and BRCA2 pathogenic variants have an emerging role for novel target therapies. BRCA1/2 have essential functions in the HR DNA repair pathway, and genomic alterations in BRCA genes lead to impaired DNA repair, called homologous recombination deficiency (HRD). Platinum compounds [such as oxaliplatin, widely used in colorectal and gastric cancer (GC) treatment] and poly (ADP-ribose) polymerase-inhibitors (PARPi) are currently the two main classes of drugs active against cancer cells harbouring HRD alterations. In particular, PARPi exposure promotes synthetic lethality in the setting of defective DNA repair pathway due to irreversible DNA damage, and resulted extremely effective in BRCA-mutant ovarian cancer (OvC): In platinum-sensitive OvC patients PARPi maintenance therapy now

represents a mainstay of treatment (Figure 1). Currently PARPi use has also been approved in BRCA-mutant breast, pancreatic and prostate cancer patients[18-21].

In BRCA-mutant GI cancer, the role of HRD alterations is still widely unknown and only few data about their clinical impact are available.

The aim of this review is to highlight a possible association between BRCA pathogenic variants and GI cancer risk and also to investigate the role of BRCA mutations as a prognostic and/or predictive factor in this setting of patients. As the role of BRCA mutations (particularly BRCA2) has already been clarified in pancreatic cancer patients and in such cases PARPi[20] are already used in everyday clinical practice, we decided to focus only on gastric, oesophageal cancer (OeC) and colorectal cancer (CRC).

LITERATURE SEARCH

The studies included in the present review were selected from PubMed/Google-Scholar/ScienceDirect databases. We used the terms "Gastric Cancer", "Colon Cancer", "Rectal Cancer", "Colorectal Cancer", "Esophageal Cancer", "Oesophageal Cancer" for tumour location and also the terms "BRCA1", "BRCA2", "BRCA" to select papers focused on BRCA mutations. "Germline" was added as a search term to identify published papers concerning familial BRCA mutations (as opposed to papers where somatic BRCA mutations or all kinds of mutations regardless of their type were included). No limitation as to the year of the published paper was used. Only published papers were included in the analysis (meeting abstracts were excluded). Duplicates were eliminated as well as articles not written in English. The search for articles was conducted independently by two of the co-authors (Maccaroni E and Giampieri R).

CRC

CRC in BRCA carriers: Risk and clinico-pathological features

CRC is the third most common malignancy in both sexes: It accounts for 10% of new cancer diagnoses and it represents the second estimated leading cause of cancer-related death in 2020 worldwide[22].

One out of 20 CRC patients has a hereditary predisposition. Lynch syndrome (LS, hereditary non-polyposis CRC) is the most common hereditary disorder, justifying 3% of all cases; LS is an autosomal dominant syndrome that causes alterations in the DNA repairing system known as mismatch repair (MMR) proteins. These proteins are able to restore insertions, deletions, as well as A-G and T-C mismatches[23]. Lack of function variants in one of the four genes (hMLH1, hMSH2, hMSH6, PMS2) that encodes the MMR proteins determines, as a final effect, an increase in replication errors in specific areas of the genome where single or double-nucleotide repeats are present, known as microsatellite regions. An increase or decrease in microsatellite length results in what is known as microsatellite instability (MSI) and increases DNA susceptibility to further mutation in oncogenes and tumour suppressor genes. Twelve to fifteen percent of all CRCs harbour a deficient MMR system and the percentage is lower (5%) if we only consider patients with metastatic involvement (mCRCs)[24]. Among these cases, 2/3 are due to sporadic mutations, while only 1/3 of cases are due to germline loss-of-function mutations. Because of this fact, patients with MSI-high status CRC diagnosis should be advised genetic counselling and testing so as not to overlook further diagnosis of LS. Other clinical factors that might lead to suspected LS are Amsterdam I/II criteria and revised Bethesda guidelines[25,26]: Although these criteria might suggest the need for pedigree analysis, it must be considered that genomic profiling remains the gold standard for diagnosis of LS either in patients who fit these criteria or those screened because of a MSI-high CRC tumour.

While the risk of developing CRC in LS is well known, the same does not apply to BRCA1/2 mutation carriers; several efforts have been made to evaluate the lifetime susceptibility of BRCA1/2 mutation carriers to develop CRC and have yielded conflicting results.

Mauri *et al*[27] suggested that BRCA1/2 mutations might determine an increase in CRC diagnoses, particularly in young patients.

Another study of Kim *et al*[28] suggested that even first and second-degree relatives of high-risk BRCA mutated (BRCAm) breast cancer patients are at increased risk of

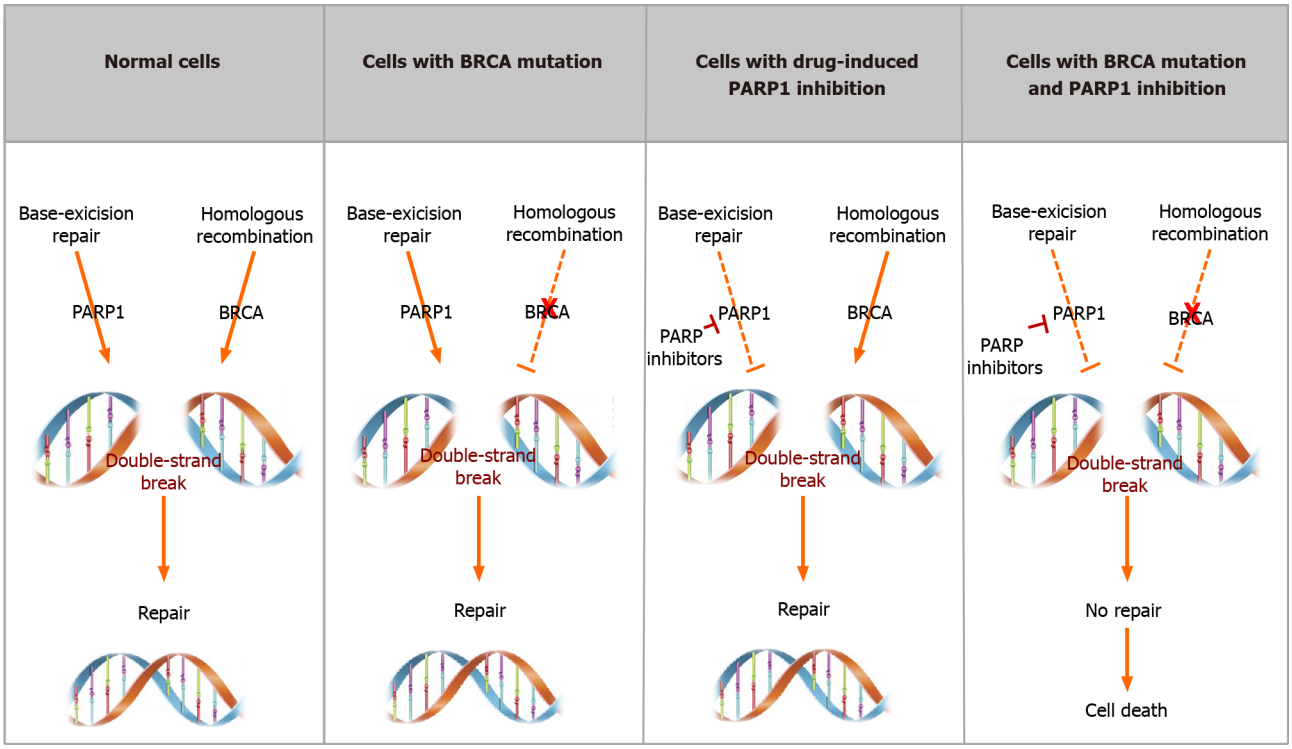


Figure 1 BRCA, PARP-inhibitors and synthetic lethality in gastrointestinal cancers.

non-breast/ovarian cancer.

Phelan *et al*[17] conducted a multicentre prospective study involving a cohort of 7105 female patients with the aim of assessing the CRC incidence in women carrying pathogenic BRCA1/2 mutations. Enrolment was conducted in fifty centres among five countries in Canada, United States and Europe, from 1992 to 2010; the median follow-up was 5.5 years.

When newly diagnosed CRC were matched with population-specific incidence rates, no statistically significant differences were found: Authors reported 21 new CRC diagnoses between mutation carriers, while 23.6 cases were expected. Among them, 16 cases occurred in BRCA1m (*vs* 17.4 expected), and 5 cases in BRCA2m (*vs* 6.1 expected). Although this study failed to show a higher prevalence in all mutated patients, subgroup analysis of BRCA1 carriers showed that early-onset (30-49 years) CRC diagnosis was four times greater than controls. The large population of patients enrolled and the prospective design of this study support these findings; however, the lack of male patients narrows these results to only the female population. Moreover, the relatively small number of incident cases and the ability to retrieve only 2/3 of the pathological reports, may represent another limitation of the study.

Similarly, Oh *et al*[29] presented a meta-analysis of 14 out of 18 studies initially selected, as to estimate the CRC risk in BRCAm carriers. Using a random-effects model, the authors observed a statistically significant increase in the odds of CRC in BRCAm carriers [odd ratio (OR) = 1.24, 95%CI: 1.02 to 1.51, P = 0.03]. When the authors focused on specific BRCA1 or BRCA2 carriers, they highlighted that BRCA1 carriers were at a higher risk of developing CRC. On the other hand, they did not observe a higher risk of early onset CRC as previously stated by Phelan *et al*[17].

In contrast, in their systematic review and meta-analysis Cullinane *et al*[30] did not observe any statistically significant difference in terms of CRC risk among BRCAm carriers, regardless of the gene involved. Neither did adjustment for age modify these findings, nor when the analysis was conducted only in the Ashkenazi Jewish heritage population.

It is well established that BRCA1/2 mutations incidence is broader in some ethnic groups, such as the Ashkenazi Jewish population; in this ethnicity there is a high population frequency (approximately 2%) of three founder mutations: BRCA1 185delAG, BRCA1 5382insC, and BRCA2 6174delT[31]. Kirchhoff *et al*[31] collected data from 586 Ashkenazi Jewish patients with pathologically confirmed diagnosis of CRC and from 5012 healthy subjects (controls), to assess whether having one of the three founder mutations could translate into an increased CRC risk. Mutations

incidence in the two groups was matched and after adjusting for age at diagnosis and sex, it was observed that harbouring BRCA1/2 mutations was not associated with higher CRC risk[31].

Similarly, Niel *et al*[32], assessed whether positive breast cancer family history could represent a risk factor for developing other tumour types. The authors performed a case-control study comparing data from 1422 patients with pathologically confirmed CRC, coming from five major hospitals in northern Israel, with 1585 control subjects. They observed that neither BRCA1/2 founder mutations nor breast cancer family history were significantly correlated with increased CRC risk.

Another interesting topic to analyse is whether BRCA-related CRC could be characterised by peculiar clinico-pathological features. Xu *et al*[33] described CRC phenotype differences among suspected LS patients with MMR and BRCA/BRCA-like carrying variants. Among 22833 patients receiving radical surgery for cancer at Fudan University Shanghai Cancer Center, 202 underwent multigene testing covering 139 genes: 42 were carriers of a pathogenic MMR-gene variant, while 20 carried BRCA or a BRCA-like variant. The most relevant differences between the two groups of patients were that the mean CRC age at diagnosis was lower in the MMR group (44.95 years *vs* 56.45 years); furthermore, BRCA/BRCA-like variants were associated with a significantly lower percentage of poorly differentiated CRC (5% *vs* 33%) and lower metachronous CRCs. Moreover, tumour sidedness was different between the two groups, with more left sided colon cancer diagnoses in MMR-gene variants, whereas extra-CRC was significantly higher in BRCA/BRCA-like families. Finally, supposed LS patients carrying BRCA and BRCA-like variants had a longer progression free survival (PFS) compared with the others[33].

Based on histological and molecular features, CRC is actually a heterogeneous group of tumours, characterised by different prognosis and response to medical treatment. Mucinous carcinoma (MC) and adenocarcinoma (AC) are the two most common histological subtypes, accounting for 10%-15% and 85%-90% of cases respectively. In particular, MC is usually associated with worse prognosis: It is more frequently diagnosed in young patients and it is usually diagnosed later than AC, due to the late occurrence of symptoms; most of these tumours occur in the proximal colon. MC is also described as having quicker disease progression. Harpaz *et al*[34] in their paper assessed the correlation between MC and BRCAm CRCs.

One thousand one hundred thirty-four mCRCs were analyzed: A statistically significant higher incidence of BRCA1/2 mutations in MC than in AC patients was observed (14.8% *vs* 4.1%, respectively). Moreover, when considering the "mutation count", defined as "somatic non-synonymous variants in encoding genes by exome sequencing" for each sample supplied by the database, they also observed that somatic BRCAm CRC has a higher mutation count than wild-type BRCA (BRCAwt); mucinous histology was also the strongest predictor of mutation count, independently of MSI as well as other variables too.

The authors conducted a prospective case-control study to confirm the results of their previous retrospective study: MC and AC CRC patients treated at Hadassah Medical Center in Jerusalem were prospectively enrolled. Thirty out of 53 MC cases were included, together with 40 controls: Authors observed a higher frequency of BRCAm patients in MC (40% *vs* 27%, respectively, $P = 0.2705$ by chi-squared test). The difference was not statistically significant. Lack of concordance with their previous results might be due to the small cohort and to other limitations: In particular, mutation analysis revealed that variants frequently seen in MC histology such as KRAS, BRAF and PI3K mutations were not observed in this cohort of patients. However, analyzing the BRCA2m group, they noted a trend toward higher frequency of mucinous histology. Moreover, they confirmed the higher tumour mutational burden (TMB) in BRCAm compared to BRCAwt carriers.

While the effects of BRCAm are well known in females, studies focused on males are rarer; indeed, it has been hypothesised that male carriers of BRCA mutations are more likely to develop several types of malignancies, including melanomas, breast, prostatic and pancreatic cancer. However, clinicians used to test far fewer male than female patients for BRCAm, thus limiting our actual knowledge of clinic-pathological and prognostic implications of these kinds of tumours.

Sun *et al*[35] conducted a retrospective pan-tumour survey to identify tumour characteristics in BRCA1/2 male mutation carriers; clinical data of mutated male patients was compared with female patients carrying the same mutation, as well as BRCAwt male patients. Results of the analysis showed that CRC incidence was higher in BRCAm males than in BRCAm female patients and non-BRCAm males. In particular, they found that deleterious mutations in the BRCA2 gene were most frequent in CRC patients. Moreover, women carrying BRCA mutations have better overall

survival (OS) and PFS than BRCAm males, principally due to the differences in tumour types and the therapeutic chances. Finally, males with deleterious BRCAm have increased OS compared to non-BRCAm.

Emerging therapeutic options in BRCA-mutated CRC

Five-years OS rate for patients with mCRC remains poor, around 14%. Standard chemotherapy for these patients includes 5-fluorouracil (5-FU) in association with irinotecan (FOLFIRI), oxaliplatin (FOLFOX) or both (FOLFOXIRI). These regimens are usually associated, particularly in first and second-line settings, with anti-EGFR or anti-VEGF targeted drugs, on the basis of tumour BRAF/RAS mutational status[36].

It has been previously described that BRCA1/2m cancers should have increased sensitivity to platinum compounds; on this basis, a few published case reports suggest that BRCA1/2 mutational testing, particularly in younger CRC patients might guide treatment selection in earlier settings of the disease. Soyano *et al*[37] described the case of a young man with locally advanced rectal cancer [T3N1M0 according to tumour-node-metastasis (TNM) staging]: He was tested for BRCA1/2 mutation and was found to be a BRCA1m carrier. After multidisciplinary evaluation, he started standard chemoradiotherapy with capecitabine as a radiosensitizer; however, when a BRCA1 mutation was found, oxaliplatin was added to standard chemoradiotherapy. It was also decided to opt for a total neoadjuvant strategy as in giving additional two cycles of chemotherapy (mFOLFOX6), after the end of chemoradiotherapy, just before surgery. The pathological report after surgery revealed complete pathological response.

Likewise, Lin *et al*[38] described the case of a 25 years old man affected with locally advanced (cT3N2M0) rectal cancer who had an excellent response to oxaliplatin-based chemotherapy. The patient was due to receive neoadjuvant concurrent standard radiochemotherapy, but given the increased risk of radiation-induced infertility he refused any radiotherapy; 4 cycles of chemotherapy 5-FU and oxaliplatin-based (FOLFOX6) were then administered. After surgical primary tumour resection performed by laparoscopic total mesorectal excision (TME), the histological report showed complete response. Owing to this excellent but unexpected reaction to oxaliplatin-based treatment, the authors decided to perform next-generation sequencing in both DNA samples taken from the primary tumour sample and the peripheral blood sample; somatic BRCA2 variant was found, as well as high TMB.

This data suggest that the assessment of BRCA-status might be performed when a particularly deep response to platinum-based chemotherapy is reached.

It has already been explained how, in cells that harbour HR system mutations leading to defective DNA double-strand break repair, as in BRCAm patients, genome stability is maintained through the activity of BER and PARP enzymes. However, Paviolo *et al*[39] demonstrated that in PARPi-treated BRCA-defective CRC samples, even though DSBs are generated, they are not directly responsible for cell death. Indeed, more than DSBs, it was proven that rapid and anomalous repair of these defects, through means of DNA repair mechanisms different from HRD, leads to severe chromosomic aberrations which are the cause of cell death.

In clinical practice, several PARPi, such as Olaparib, Niraparib, Rucaparib, are widely used in treating ovarian, breast, pancreatic and prostate cancer harbouring BRCA mutations or HRD signature, and they are under investigation in many other neoplasms.

Veliparib (ABT-888) was the first PARPi to be studied in CRC treatment: Prior works demonstrated its efficacy in BRCA-deficient compared to proficient cells and proved that combining platinum-based compounds with PARPi provided increased response[40]. Harpaz *et al*[34] described the use of Veliparib as maintenance therapy in a patient carrying a germline pathogenic BRCA1m, affected by metastatic KRAS and BRAF wild type rectal adenocarcinoma with pelvic and lung metastases. He underwent first-line chemotherapy with FOLFOX and Panitumumab, and obtained maximal response. Then he started maintenance veliparib, for as long as 2 years; treatment was then stopped after occurrence of new metastases.

Niraparib, a potent and selective PARP1/2i, has shown *in vitro* and *in vivo* efficacy in BRCA mutant cell lines; sensitivity to Niraparib monotherapy is 10-fold in BRCA mutant cell lines compared to BRCAwt cells[41].

A phase 2, multicentre, open-label study evaluating Rucaparib, an orally available PARP1i, in patients with unresectable, locally advanced or metastatic solid tumours with deleterious mutations in HR repair genes is currently still recruiting; the trial enrols patients with either somatic or germline variants and BRCA1 and BRCA2 mutations are allowed. (NCT04171700). Primary outcome is the best overall response rate[42].

In addition to that, a clinical trial evaluating the use of immune checkpoint inhibitors in solid tumours with BRCA mutation is still ongoing[43] (Table 1).

Despite these premises, at the moment there is still no evidence-based data that supports the use of these drugs in CRC patients; because of this it is suggested that PARPi-based clinical trials should be enforced in BRCAm carriers to further validate their use in this setting.

Furthermore, whole-genome sequencing in BRCA1/2m patients allowed identification of different signatures correlated with BRCA status; this BRCA-ness signature was present even in cells without BRCA1/2 mutations, suggesting that the signature might be more likely to predict HRD compared with simple assessment of BRCA mutational status[40]. Should the signature be more widely used, it can be expected that it will be tested to predict PARPi sensitivity.

GC

Worldwide, GC is one of the most common malignancies and the third leading cause of cancer-related mortality[44]. Approximately 8%-30% of patients have a positive family history[45], but only 1%-3% of GCs are truly hereditary[46]. GC predisposition has been linked to familial cancer syndromes, including hereditary diffuse GC[47], LS [48], Peutz-Jeghers syndrome[49], Li-Fraumeni syndrome[50], familial adenomatous polyposis syndrome[51], adenocarcinoma and proximal polyposis syndrome of the stomach[52].

The association between germline mutation of BRCA genes and risk of GC was investigated in several studies, although some aspects remain unclear.

Historically, carriers of germline BRCA1/2 mutations have shown a four to six-fold increased risk of developing GC compared to the general population[53,54] and the risk appears to be higher in patients with BRCA1 mutations than in patients with BRCA2 mutations[54,55].

Regarding BRCA2 carriers, the Polish study by Jakubowska *et al*[56] assessed the importance of a family history of GC to predict the presence of BRCA2 mutations in ovarian cancer patients. In this study, BRCA2 mutation was found in 8 of 34 women with ovarian cancer and a family history of GC *vs* 3 of 75 women with OR and a family history of ovarian cancer, but not of GC (OR = 7.4; 95%CI: 1.8-30; P = 0.004); this finding would confirm that GC is a BRCA2-related tumour.

Remarkably, a study conducted by the same group of authors, suggested that founder BRCA1 mutations reported in Polish breast/ovarian cancer patients do not contribute to increased GC risk[57].

Furthermore, Lorenzo Bermejo *et al*[58] demonstrated that GC before the age of 70 was twice as frequent in families with breast and ovarian cancers as in the general population; similarly, Schlebusch *et al*[59] found a high prevalence of GC in the BRCA2-positive families compared with the general population.

Nevertheless, in a study performed in the Netherlands, 139 BRCA2 families with 66 different pathogenic mutations were analysed and significantly increased risk of developing GC was not observed[16].

These conflicting data could be partially justified by the high prevalence of Ashkenazi Jews in the studies of Schlebusch *et al*[59] and Jakubowska *et al*[56]; in fact, ovarian, pancreatic and gastric cancer as well as non-Hodgkin's lymphoma have a higher incidence rate in the Ashkenazi population[60].

To further assess the impact of founder mutations in the Ashkenazi population, Figer *et al*[61] focused on a founder BRCA2 variant that is present in about 1.5% of the general Ashkenazi population and rarely in non-Ashkenazi Jews. In order to evaluate the contribution of this mutation to non-CRC GI cancer, they tested 70 consecutive unselected Ashkenazi Jews with GI malignancies that carried this specific mutation. They concluded that the rate of the Ashkenazi Jews with BRCA2 founder mutation in patients with GC was 5.7%, approximately five times higher than the general population, supporting the hypothesis of an increased risk of GC in BRCA2-carriers[46].

It has previously been reported that GC in BRCA2 carriers may be sex-related, as it seems to be more frequent in males[6,58]; in an interesting article, Cavanagh *et al*[62] argue that women carrying BRCA1/2 mutations usually develop early-onset breast and ovarian cancer and therefore may not survive long enough to develop GC at an older age; this would explain the high prevalence of GC (and other cancers) in male BRCA-carriers.

Regarding BRCA-associated tumours in males, Sun *et al*[35] recently conducted a retrospective pan-tumour survey on 346 cases of BRCA-associated tumours including

Table 1 Ongoing trials including patients carrying gastro-intestinal cancer with BRCA1m and/or BRCA2m and solid tumours with BRCA1m and/or BRCA2m

	Study title	Design	Status	Trial description
NCT03337087	Liposomal irinotecan, fluorouracil, leucovorin calcium, and rucaparib in treating patients with metastatic pancreatic, colorectal, gastroesophageal, or biliary cancer	Phase I	Recruiting	Experimental: nal-IRI, leucovorin, fluorouracil, rucaparib
NCT02286687	Talazoparib in treating patients with recurrent, refractory, advanced, or metastatic cancers and alterations in the BRCA genes	Phase IIa	Recruiting	Experimental: Talazoparib
NCT03428802	Pembrolizumab in treating participants with metastatic, recurrent or locally advanced cancer and genomic instability	Phase IIa	Recruiting	Experimental: Pembrolizumab
NCT04503265	A trial of AMXI-5001 for treatment in patients with advanced malignancies	Phase I-IIa	Recruiting	Experimental: AMXI-5001 (oral PARP and microtubule polymerization inhibitor)
NCT04171700	A study to evaluate rucaparib in patients with solid tumors and with deleterious mutations in HRR genes (LODESTAR)	Phase IIa	Recruiting	Experimental: Rucaparib
NCT03415659	Phase I clinical study of HWH340 tablet in patients with advanced solid tumors	Phase I	Recruiting	Experimental: HWH340 monotherapy
NCT02723864	Veliparib (ABT-888), an Oral PARP inhibitor, and VX-970, an ATR inhibitor, in combination with cisplatin in people with refractory solid tumors	Phase I	Active, not recruiting	Experimental: VX-970 at Days 2 and 9 of each 21-d cycle; veliparib twice a day (BID) days 1-3 and 8-10 of each cycle; cisplatin at day 1 (and day 8 from DL3 onwards) of each cycle
NCT00516373	A study to assess the safety and pharmacokinetics of an inhibitor of Poly ADP-Ribose Polymerase-1 (PARP)	Phase I	Active, not recruiting	Experimental: KU-0059436 (oral PARP inhibitor)
NCT04439227	Testing AZD1775 as a potential targeted treatment in cancers with BRCA genetic changes (MATCH-Subprotocol Z1I)	Phase II	Active, not recruiting	Experimental: Adavosertib
NCT03565991	Javelin BRCA/ATM: Avelumab plus talazoparib in patients with BRCA or ATM mutant solid tumors	Phase II	Active, not recruiting	Experimental: Combination of avelumab and talazoparib
NCT01482715	A study of oral rucaparib in patients with a solid tumor (Phase I) or with gBRCA mutation ovarian cancer (Phase II)	Phase I-IIa	Completed	Experimental: Rucaparib
NCT01989546	Pilot trial of BMN 673, an oral PARP Inhibitor, in patients with advanced solid tumors and deleterious BRCA mutations	Phase I-IIa	Completed	Experimental: Talazoparib (BMN 673)
NCT01286987	Study of Talazoparib, a PARP Inhibitor, in patients with advanced or recurrent solid tumors	Phase I	Completed	Experimental: Talazoparib
NCT03767075	A modular multi-basket trial to improve personalized medicine in cancer patients (BoB)	Phase II	Recruiting	Experimental: Atezolizumab

nal-IRI: Liposomial Irinotecan; PARP: Poly-ADP ribose polymerase; ATR: Ataxia telangiectasia and Rad3-related protein; ATM: Ataxia telangiectasia mutated; BID: Bis in die; DL3: Dose level 3; BoB: Basket of baskets.

GC in males and a comparative analysis among male and female BRCA carriers (349 cases), as well as in male patients who were not BRCA carriers (4577 cases); similar incidences of BRCA mutations (6.0% *vs* 6.6%) and age at diagnosis of BRCA-related tumour (median, 65 *vs* 60 years) were observed both in male and female patients.

Moreover, when evaluating both OS and PFS in patients who developed GC, interesting differences were observed: Compared with females, BRCA mutations in males were associated with decreased OS and PFS; however, subgroup analysis demonstrated that BRCA mutation was associated with increased OS in GC (HR for OS: 0.60 $P = 0.05$). Considering the limited data about the outcome of gastric cancer in BRCA carriers, these results are of particular note. However, data regarding the prognostic impact of BRCA pathogenic variants in GC patients are still scarce.

Another study supporting these results was conducted by Halpern *et al*[63], where ten GC patients with BRCA mutations were assessed; 6/10 patients had metastatic disease. Median OS of all ten GC patients was 47.5 mo. Median OS for patients diagnosed with operable disease was 55.5 mo and was 32 mo for patients with metastatic disease. Particularly, patients with metastatic disease have a 1-, 2- and 3-

year survival rate of 100%, 83.3% and 50%, respectively. Albeit the number of patients included in the analysis is rather limited, it is noteworthy, as it seems to support the idea that BRCA mutations might be associated with better survival: Nowadays median OS of patients with metastatic gastric cancer in western countries is usually around 10-12 mo.

In order to clarify the real incidence and outcome in BRCA patients with GC, a trial is currently active; in this prospective trial the investigators aim to evaluate the incidence of BRCA loss in patients with advanced GC and to observe the treatment outcome and the possibility of BRCA loss as a predictive and prognostic factor[64].

As previously discussed, existing clinical studies have suggested that some BRCA-associated tumours are sensitive to PARPi[18]. Therefore, a phase I/II study investigating side effects and best dose of lyposomal irinotecan and rucaparib when given together with fluorouracil and leucovorin calcium is currently available for BRCA patients with pancreatic, colorectal, gastroesophageal or respiratory cancer biliary.

Other basket trials available for patients with BRCA mutations are summarised in Table 1.

Finally, several cancers in BRCA carriers have been shown to be particularly sensitive to platinum-based chemotherapy[65,66], but studies evaluating the chemosensitivity of patients with BRCA germline mutations and GC are not available.

However, previous clinical and preclinical studies have reported an association between a low level of BRCA1 expression or a BRCA1 mutation and the chemosensitivity and prognosis of sporadic GC.

According to the American Joint Committee on Cancer, patients with a negative-BRCA1 sporadic GC are more likely to have a high grade of tumour, a high TNM stage and a poorly differentiated tumour[53,67]; at the same time, other studies have revealed that BRCAnegative sporadic GC are more sensitive to platinumbased adjuvant chemotherapy compared with BRCA1positive sporadic GC[68]. These findings indicate that patients with BRCA1negative GC have a longer OS time and improved prognosis, which suggests an important association between BRCA1 expression and platinumbased chemotherapy.

OEC

OeC is the seventh most common cancer and the sixth leading cause of cancer-related deaths worldwide[69]. The incidence rate of OeC varies considering the location; particularly high frequency is present in East Asia and Eastern/Southern Africa, where oesophageal squamous cell carcinoma (SCC) represents the main histology, while adenocarcinoma is more common in Western countries[70]. Most cases of OeC are sporadic and caused by somatic mutations[71] and oesophageal lesions are rarely described in hereditary CRC syndromes as LS or familial adenomatous polyposis (FAP). However, several studies reported the occurrence of OeC in patients with attenuated FAP or Gardner's syndrome, which are both caused by mutations in the *APC* gene[72]. The association between BRCA1/2 germline mutations and OeC has been explored in some studies.

Moran *et al*[73], in a family-based study, observed a relative risk increase of OeC (regardless of histology) (relative risk 2.9, 95%CI: 1.1-6) in families with BRCA1 mutations.

Conversely, BRCA2 carriers seem to have a higher risk of developing SCC than Adenocarcinoma.

In fact, contribution of BRCA2 mutations for the development of SCC has been reported in high- and low-risk Chinese populations, in the Turkmen population of Iran and in the very high risk region of northeast India. Specifically, Hu *et al*[74] have reported five different BRCA2 mutations in 6 out of 44 (13%) patients with SCC in high-risk Chinese population, while Akbari *et al*[75] observed a nonsense BRCA2 pathogenic gene variant in eight SCC cases with a family history of OeC in the Turkmen population of Iran. Instead, Kaushal *et al*[76] have investigated 317 cases of SCC in a high-risk region of India and they conducted, performing *BRCA2* gene germline mutations, screening in 20 familial and 80 non-familial SCC patients: They found non-synonymous BRCA2 variants in 3 out of 20 patients with familial SCC, while no sequence alterations were found in 80 non-familial SCC cases. Moreover, a study to screen mutations of *BRCA2* gene in 47 SCC patients from a low-risk Chinese population was conducted by Zhong *et al*[77]. They found 9 germline missense point mutations in apparently sporadic male patients, with a mutation frequency of 19%. In addition to that, when an additional cohort of 94 healthy controls underwent screening

for the 9 mutations previously identified in SCC cases, only 2 positive individuals (mutation frequency 2%) were found.

Finally, a recent, large-scale study by Ko *et al*[78] included 4517 individuals: 186 familial SCC patients from a high risk region of China, while the rest were healthy East Asian individuals (3289 Henan and 1228 moderate-risk Hong Kong Chinese). They identified BRCA2 Loss-of-function mutations in 3.23% (6/186) familial SCC patients compared to 0.21% in the East Asians (OR = 15.89, $P = 2.48 \times 10^{-10}$).

Regarding the therapeutic implications between BRCA carriers and OeC, few data are still available in the literature. However, encouraging preclinical studies would show that PARPi in combination with a DNA damaging agent might be beneficial in this setting[79]. Furthermore, the PARPi olaparib appears to sensitise OeC cells to fractionated proton irradiation[80].

DISCUSSION

Since the discovery in the mid-nineties of both *BRCA1/2* genes and their impact on breast and ovarian cancer risk, both surveillance and risk-reducing surgical procedures have been developed as effective means to reduce the risk of developing both malignancies and to overall reduce the risk of death in carriers of these variants. However, recently, BRCA carriers have been recognised to be at risk of developing other kinds of cancer types, such as prostate, pancreatic cancer and melanoma[10-14].

GI cancers are a heterogenous group of malignancies that, taken together, represent the most common tumour type worldwide. Most tumours of this group are mainly due to environmental and lifestyle risk factors, while the weight of hereditary predisposition is rather low (usually around 5%-10% of all cancers). LS represents the most common form of hereditary predisposition to these kinds of tumours (mainly for CRC), while the role of BRCA1/2 mutations is less understood.

In our review we tried to summarize the current published evidence on the role of BRCA1/2 pathogenic variants in colorectal, gastric and OeC risk and prognosis.

As for CRC, there is no definitive consensus regarding increased risk of development of this malignancy: Some studies[17,29] seem to suggest that there might be an increased incidence of CRC in BRCA mutation carriers, particularly for BRCA1 carriers. In those studies where a positive correlation with increased risk of CRC development was proven, a trend towards younger age of onset was also suggested. On the other hand, other studies[30,31] have failed to confirm these findings, also in selected high-risk populations where the presence of BRCA variants is higher than in the general population (such as Ashkenazi Jews). Both these studies have some limitations that make direct comparison impossible: In the study of Phelan *et al*[17] the population consists entirely of female carriers, thus narrowing their findings to women. Conversely, the negative results of the studies of Kirchhoff and Bethany, conducted in the high-risk Ashkenazi Jewish population, might be influenced by the relatively higher risk of developing CRC in this ethnic group compared to people of other ethnicities[31,32,81].

While there is no consensus on the impact of germline BRCA1/2 mutations on CRC risk, their prognostic role is more clearly defined: A series of studies have shown how, in patients with CRC, BRCA mutations might increase the likelihood of response to chemotherapy. The evidence is, however, mainly derived from case reports or small retrospective case-control studies where patients are treated with oxaliplatin-based chemotherapy. All these studies suffer from selection biases, as only patients who had particularly significant responses to oxaliplatin-based treatment (and that should have more favourable prognosis), underwent genetic testing; there is a general lack of information concerning whether BRCA mutations may influence CRC prognosis regardless of treatment received. It is hoped that, in the near future, wide screening with multi-panel molecular testing in CRC patients will give us a clearer estimate of the real prevalence and prognosis of BRCA mutations; as multi-panel molecular testing will be used more frequently as a means to identify those patients who will be eligible to receive specific targeted drugs, and we will be able to identify more accurately people that might be carriers of germline BRCA mutations that would not be suspected on the basis of their family history.

The role of BRCA mutations in gastric cancer seems to be more clearly defined compared to CRC: A few studies have indicated an increased risk of gastric cancer in families with either BRCA1 or BRCA2 mutations. Even though few papers[54] suggest higher risk in BRCA1 carriers compared to BRCA2, most papers seem to agree that BRCA2 mutations are more clearly associated with an increased GC risk[56,58,59]. As

well as in CRC, most studies that have shown a positive impact of BRCA mutations on GC risk have also shown an increased likelihood of early onset (< 70 years old) GC.

As stated before, all those studies that have enrolled specific ethnic groups that have a higher risk of harbouring BRCA mutations (such as Ashkenazi jews) have met with negative results; however, when the analysis was conducted by taking into account this factor, BRCA mutations maintained their causative role. Interestingly, most data that have focused on the prognosis of GC patients harbouring BRCA mutations agree on a positive prognostic role: This might partly be due to the fact that standard treatment of gastric cancer patients includes platinum compounds; it would be interesting to assess whether GC patients who harbour BRCA mutations and that do not receive any kind of platinum-based treatment (as those treated with fluoropyrimidines alone) retain the same positive prognosis.

Surprisingly, although OeC is usually considered as a cancer type where environmental and lifestyle factors predominantly influence the risk of the onset of disease, a lot of published evidence can be found concerning the role of BRCA mutations in this disease. Out of the two main histologies, SCC seems to be much more closely associated with BRCA mutations compared with adenocarcinoma. All papers seem to suggest that BRCA2 mutations are more frequently observed in this tumour type compared with BRCA1 mutations. Although studies that focused on the prognostic or predictive role of BRCA1/2 are lacking, it is expected that, based on the standard treatment options for this kind of disease that include platinum compounds and radiotherapy, more favourable outcome for patients who are carriers of these mutations can be expected.

The retrospective nature of these studies represents a major limitation; it is however interesting to notice that most of them indicate a positive correlation of germline BRCA1/2 mutations and an increased GI cancer risk. BRCA1/2 variant assessment will soon become one of the most promising fields of research in these kinds of diseases: Most of these tumours are treated with platinum compounds (either Oxaliplatin or Cisplatin) and it is expected that BRCA mutation carriers should have greater likelihood of response to treatment compared to wild type BRCA individuals. Furthermore, as in pancreatic cancer where PARPi have now become the standard care for patients with BRCA germline mutations[20], we can expect that the same might apply to other cancers arising from other areas of the GI tract: Indeed, PARPi are currently being evaluated in a wide number of clinical trials (Table 1).

In addition to that, it is actually recognised that, in CRC, GC and OeC, completely different clinical presentations are seen: While older patients have experienced in the last decade an improvement in survival (mainly due to introduction of screening procedures and advances in adjuvant therapy[36], younger patients are still those whose survival has not changed; most papers that have focused on BRCA1/2 mutations came to the conclusion that BRCA germline carriers are those who have the highest likelihood of early onset GI presentation. We believe that further research on early onset GI cancer might identify a greater number of patients where germline defects rather than environmental risk factors are the main causes of disease; this might also be associated with differences in treatment response. Moreover, it would advocate different surveillance procedures, as younger patients are not usually taken into account in most screening programmes.

CONCLUSION

In conclusion, we believe that this review has highlighted the importance of BRCA1/2 germline mutations as risk factors in GI malignancies and has drawn attention to future applications of this knowledge in clinical practice.

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REVIEW

GOECP/SEOR clinical guidelines on radiotherapy for malignant pleural mesothelioma

Javier Luna, Andrea Bobo, Joaquín José Cabrera-Rodriguez, María Pagola, Margarita Martín-Martín, María Ángeles González Ruiz, Miguel Montijano, Aurora Rodríguez, Lira Pelari-Mici, Almudena Corbacho, Marta Moreno, Felipe Couñago

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Abstract

Malignant pleural mesothelioma (MPM) is a rare tumor with poor prognosis and rising incidence. Palliative care is common in MPM as radical treatment with curative intent is often not possible due to metastasis or extensive locoregional

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involvement. Numerous therapeutic advances have been made in recent years, including the use of less aggressive surgical techniques associated with lower morbidity and mortality (*e.g.*, pleurectomy/decortication), technological advancements in the field of radiotherapy (intensity-modulated radiotherapy, image-guided radiotherapy, stereotactic body radiotherapy, proton therapy), and developments in systemic therapies (chemotherapy and immunotherapy). These improvements have had as yet only a modest effect on local control and survival. Advances in the management of MPM and standardization of care are hampered by the evidence to date, limited by high heterogeneity among studies and small sample sizes. In this clinical guideline prepared by the oncological group for the study of lung cancer of the Spanish Society of Radiation Oncology, we review clinical, histologic, and therapeutic aspects of MPM, with a particular focus on all aspects relating to radiotherapy, including the current evidence base, associations with chemotherapy and surgery, treatment volumes and planning, technological advances, and reradiation.

Key Words: Malignant pleural mesothelioma; Chemotherapy; Surgery; Radiotherapy; Radiation techniques; Reradiation

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Core Tip: Malignant pleural mesothelioma is a rare tumor that is very challenging to treat. Technological advances in surgery and radiotherapy are largely responsible for the marginally improved outcomes observed in recent years. Heterogeneity among studies and a lack of phase III randomized controlled trials are some of the main barriers to achieving more effective, standardized treatments. In this review article, we provide an in-depth analysis of changes in the clinical, histologic, and therapeutic profile of malignant pleural mesothelioma in recent decades and highlight the main research areas in this field.

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INTRODUCTION

Malignant pleural mesothelioma (MPM) is a rare tumor, but it is a global health concern because of its poor prognosis and rising incidence.

Improvements in our understanding of MPM pathogenesis[1,2], together with encouraging results from recent studies of multimodality therapies, targeted therapies, and immunotherapies have brought new hope for the management of this disease[3].

Optimal treatment strategies have not been defined for MPM. In these guidelines, drawn up by the Oncological Group for the Study of Lung Cancer of the Spanish Society of Radiation Oncology, we review the current status and prospects of MPM management with a focus on all aspects relating to radiotherapy.

Epidemiology

Incidence: MPM incidence is strongly associated with asbestos exposure[4,5], but there is a long latency period, meaning that in some cases, the tumor can take up to 40 years to appear. Projections from the end of the last century suggested that the incidence of MPM in Western Europe would peak around 2020[6], and recent epidemiological data would appear to confirm this trend. MPM incidence rates are lower in some parts of Asia and Central and Eastern Europe, but this could be due to less rigorous data collection or reporting[7] or higher mortality due to other causes. In short, based on the available data, MPM incidence and mortality rates vary consid-

erably according to geographic location.

Etiology

Asbestos: Asbestos is the main cause of MPM. This mineral was widely used for many years in construction products (*e.g.*, roofing and tiles), friction materials, packaging, textiles, paint, and a range of other industrial products.

Although the association between asbestos exposure and MPM has been demonstrated[8], it has not been possible to define cumulative exposure limits, meaning that anyone exposed to asbestos is potentially at risk.

Occupational exposure accounts for over 80% of MPM cases in men[9-12], explaining differences in attributable risk between men and women.

Other minerals: Other elongated mineral particles such as erionite[13,14] and fluoro-edenite[15] may also have a causative role in MPM. Environmental exposure to these minerals is higher in certain countries, such as Turkey, the United States, and Mexico [16-18].

Genetic predisposition: Familial aggregation studies have also reported an increased risk of MPM among offspring and siblings of patients with the disease[19-21]. This increased risk has been linked to a germline mutation in *BAP-1* (breast cancer gene 1-associated protein), which is a tumor suppressor gene with a role in DNA transcription and repair[22,23].

Germline mutations in cancer-susceptibility genes have been reported in a significant proportion of MPM patients, particularly those with peritoneal mesothelioma, younger patients, patients with minimal asbestos exposure, and those with a second cancer[24,25].

Treatment

The treatment options for MPM are largely determined by stage, and approaches vary depending on whether the patient has operable or inoperable disease and is deemed suitable for surgery. Surgery, radiotherapy, and systemic therapies are constantly evolving to meet better the challenges associated with each stage of disease.

Hemithoracic radiotherapy as an adjunct to pneumonectomy has been used in MPM for years, but its effect on disease control remains modest. As a palliative measure, it has proven effective at improving pain and quality of life.

More advanced radiotherapy techniques [respiratory control, four-dimensional imaging, intensity-modulated radiotherapy (IMRT), stereotactic body radiation therapy (SBRT), and proton therapy] have since emerged and are used in combination with induction chemotherapy and/or surgery or as radical therapy in patients with unresectable disease.

Radiotherapy has also been proposed as a salvage strategy for patients whose disease recurs following surgery or chemotherapy. Its role for improving progression-free and overall survival (OS) in patients with oligoprogressive disease is also being investigated.

METHODOLOGY: LEVELS OF EVIDENCE

As mentioned, no optimal treatment strategies have yet been established for MPM, and recent clinical guidelines offer contrasting conclusions and recommendations, even though they are based on the same scientific evidence. These guidelines include the British Thoracic Society Guideline for the Investigation and Management of Malignant Pleural Mesothelioma[26], the National Comprehensive Cancer Network Malignant Pleural Mesothelioma Guidelines[27], the American Society of Clinical Oncology Clinical Practice Guideline for the Treatment of Malignant Pleural Mesothelioma[28], and the European Respiratory Society/European Society of Thoracic Surgeons/European Association for Cardio-Thoracic Surgery/European Society for Radiotherapy and Oncology Guidelines for the Management of Malignant Pleural Mesothelioma[29].

In the current clinical practice guidelines, we will apply the system used by the European Society of Medical Oncology[30] to guide decision-taking regarding the management of MPM (which in turn was adapted from the Infectious Diseases Society of America's grading system for ranking recommendations in clinical guidelines[31]). Accordingly, the strength of a given recommendation for or against a preventive or therapeutic practice is graded using the letters A-E, while the quality of the supporting evidence is graded using the Roman numerals I-III (Table 1).

Table 1 Infectious Diseases Society of America levels of evidence and grades of recommendation and European Society of Medical Oncology adaptation

IDSA United States Public Health Service Grading System for Ranking Recommendations in Clinical Guidelines		ESMO adaptation of IDSA Grading System	
Category, grade	Definition	Category, grade	Definition
Strength of recommendation		Grades of recommendation	
A	Good evidence to support recommendation for use	A	Strong evidence for efficacy with a substantial clinical benefit, strongly recommended, strongly recommended
B	Moderate evidence to support recommendation for use	B	Strong or moderate evidence for efficacy but with a limited clinical benefit, generally recommended
C	Poor evidence to support a recommendation	C	Insufficient evidence for efficacy or benefit does not outweigh the risk or the disadvantages (adverse events, costs, *etc.*), optional
D	Moderate evidence to support a recommendation against use	D	Moderate evidence against efficacy or for adverse outcome, generally not recommended
E	Good evidence to support a recommendation against use	E	Strong evidence against efficacy or for adverse outcome, never recommended
Quality of evidence		Levels of evidence	
I	Evidence from > 1 properly randomized, controlled trial	I	Evidence from at least one large randomized, controlled trial of good methodological quality (low potential for bias) or meta-analyses of well-conducted randomized trials without heterogeneity
II	Evidence from > 1 well-designed clinical trial, without randomization; from cohort or case-controlled analytic studies (preferably from > 1 center); from multiple time series; or from dramatic results from uncontrolled experiments	II	Small randomized trials or large randomized trials with a suspicion of bias (lower methodological quality) or meta-analyses of such trials or of trials with demonstrated heterogeneity
III	Evidence from opinions of respected authorities, based on clinical experience, descriptive studies, or reports of expert committees	III	Prospective cohort studies
		IV	Retrospective cohort studies or case-control studies
		V	Studies without control group, case reports, experts opinions

ESMO: European society for medical oncology; IDSA: Infectious Diseases Society of America.

The grades of recommendation and levels of evidence for the use of surgery, chemotherapy, and radiotherapy in MPM are summarized in Table 2.

DIAGNOSIS AND TUMOR, NODE, METASTASIS (TNM) STAGING

Imaging techniques

Chest radiography normally shows pleural effusion and thickening.

Computed tomography (CT) of the chest is the first-line imaging test for patients with suspected MPM. It typically shows findings suggestive of MPM, such as thickening of the mediastinal pleura[32].

Positron emission tomography (PET)-CT may be useful in certain cases but should not be used in patients who have undergone pleurodesis as this procedure can affect maximum standardized uptake values. PET-CT has low sensitivity for stage N1 (38%) and T4 (76%) disease[33]. Compared with CT alone, it has higher specificity for disease stages II (100% *vs* 77%) and III (100% *vs* 75%)[34].

Magnetic resonance imaging (MRI) has not yet been validated in MPM. It may be useful for identifying margins in patients with multifocal chest wall involvement and for demonstrating unresectability.

Diagnostic procedures

Thoracoscopy is the diagnostic technique of choice in MPM. Other less invasive biopsy techniques, however, can be used in certain cases.

Table 2 Recommendations for the treatment of malignant pleural mesothelioma using the European Society of Medical Oncology levels of evidence and grades of recommendation adapted from the Infectious Diseases Society of America

Surgery	Chemotherapy	Radiotherapy
For palliation of pleural effusions when patients cannot benefit from chest tube drainage or chemical pleurodesis or when these are not successful (II, A)	The anti-folate/platinum doublet is the only approved standard of care for the first- and second-line treatment of unresectable mesothelioma (I, A); If available, bevacizumab, could be added to the standard treatment in selected patients (II, B)	For palliation of pain related to tumor growth radiotherapy can be considered (II, A)
To obtain diagnostic samples of tumor tissue and to stage the patient (II, A)	Maintenance therapy (switch or continuation) has not yet improved overall survival and patients should be included in these studies (II, A)	The use of radiotherapy to prevent growth in drainage tracts is not proved to be useful (III, A)
To be part of a multimodality treatment, preferably as part of a study (II, A)	Patients in good condition should be recommended to join studies in second line (II, A)	Radiotherapy can be given in an adjuvant setting after surgery or chemo-surgery to reduce the local failure rate. However, no evidence is available for its use as a standard treatment (II, A)
To perform a macroscopic complete resection by means of pleurectomy/decortication (III, C)		When postoperative radiotherapy is applied, strict constraints must be adhered to in order to avoid toxicity to neighboring organs, and special, tissue sparing, techniques should be used (II, A)

ESMO: European Society for Medical Oncology; IDSA: Infectious Diseases Society of America.

Closed pleural biopsy: Closed (blind) pleural biopsy with an Abrams needle has a diagnostic sensitivity of 27%-60% for malignancy[35,36]. Complications are common, in particular pain and pneumothorax (around 15%). In the largest review to date of closed pleural biopsies (n = 2893), the diagnostic yield for malignancy was 57%[37].

Image-guided pleural biopsy: A number of studies have reported higher diagnostic sensitivity (around 88%[38]) for image-guided cutting-needle biopsy compared with closed pleural biopsy.

Maskell *et al*[39] demonstrated that CT-guided cutting-needle biopsy was approximately 40% more sensitive than Abrams biopsy for diagnosing malignancy. The technique correctly diagnosed malignancy in 13 of 15 patients with suspected malignant pleural effusions (sensitivity, 87%; specificity, 100%).

In 1999, Heilo *et al*[40] reported that ultrasound-guided core-needle biopsy had a diagnostic sensitivity of 77%, a specificity of 88%, and a positive predictive value of 100% for MPM.

Thoracoscopy: Thoracoscopy has a high diagnostic yield in pleural malignancies. In an analysis of 1369 patients from 22 case series, thoracoscopy had a diagnostic sensitivity of 92.6% for malignant pleural disease. Complication rates are very low, and a mortality rate of 0.34% has been reported[41]. Thoracoscopy has been found to have superior diagnostic sensitivity and specificity than both closed and image-guided pleural biopsy[42,43].

Video-assisted thoracoscopic surgery: Numerous case series have found video-assisted thoracoscopic surgery (VATS) to have high diagnostic sensitivity and specificity for malignant pleural disease[44,45].

Although no direct comparisons have been made of medical and surgical thoracoscopy, data suggest that the two procedures have a very similar sensitivity.

VATS pleural biopsy has a sensitivity of 95%, a specificity of 100%, and a negative predictive value of 94%[44,45].

Pathology

MPM should be diagnosed according to the 2015 World Health Organization (WHO) Classification using an adequate tissue sample.

MPM can be divided into diffuse malignant mesothelioma (DMM), localized malignant mesothelioma (LMM), and well-differentiated papillary mesothelioma (WDPM). DMM has a worse prognosis than LMM and WDPM.

DMM can be further categorized as epithelioid, sarcomatoid, or biphasic. The second two subtypes are associated with worse survival[46]. Pleomorphic epithelioid DMM is a particularly aggressive variant[47].

Cytologic examination of pleural fluid is usually the first diagnostic test, as pleural effusion is typically the first clinical sign detected. Not all epithelioid subtypes, however, cause pleural effusion, and sarcomatoid subtypes do not generally spread to the serosal cavity.

Cytology may also be useful when pleural biopsy is not possible.

The 2015 WHO classification of tumors of the lung, pleural, thymus, and heart introduced a number of changes in relation to MPM. First, it recognized that the pleomorphic subtype, like the sarcomatoid variant, is associated with poor prognosis. It also recognized the usefulness of immunohistochemistry for distinguishing between MPM and carcinoma, and redefined the criteria for differentiating MPM from reactive mesothelioma proliferations.

The discovery that the *NAB2-STAT6* fusion was a hallmark of solitary fibrous tumors had important diagnostic implications. The *WWTR1-CAMTA1* fusion is a marker of epithelioid hemangioendothelioma, while desmoid-type fibromatosis is frequently associated with *CTNNB1* mutations and beta-catenin expression.

Immunohistochemistry

Immunohistochemistry is an important diagnostic tool as it can help distinguish between epithelioid mesothelioma and other carcinomas involving the pleura (mainly lung adenocarcinoma).

Claudin 4 has emerged as one of the most useful markers for differentiating mesothelioma (claudin 4^-) from adenocarcinomas (claudin 4^+)[48].

Calretinin, cytokeratin 5/6, Wilms tumor 1, and D2-40 are all markers of mesothelioma, while carcinoembryonic antigen, B72.3, Bg8, BerEP4, and M031 are markers of carcinoma[49].

Epithelioid DMM can sometimes resemble squamous cell carcinoma, but it does not show nuclear staining with p40 (or p63)[50].

The new WHO Classification also recognizes the difference between DMM and reactive mesothelial proliferations or hyperplasia. Given the absence of specific markers for benign mesothelial processes, the distinction between benign and malignant proliferations is usually based on morphologic features.

WDPM, can be difficult to distinguish from conventional forms of DMM. It is characterized by small translucent nodules, whereas DMM usually shows solid nodules and pleural effusion or thickening. It also follows a characteristically indolent course[51].

The three best-defined genetic alterations in MPM are loss of neurofibromatosis type 2 (45%-50% of cases), homozygotic deletion of *CDKN2A* (p16) (100% of sarcomatoid cases), and loss of *BAP-1* (45%-100% of cases, most of which are epithelioid). *BAP-1* loss is associated with good prognosis, while *CDKN2A* loss is associated with poor prognosis.

Staging

Eighth edition of the TNM classification for mesothelioma (Tables 3 and 4)

The new TNM staging system for mesothelioma is summarized in Tables 3 and 4. The tables were created by the International Association for the Study of Lung Cancer (IASLC) and are published in the eighth edition of the American Joint Committee on Cancer Staging Manual[52-54].

CLINICAL INDICATIONS BY TUMOR STAGE AND TREATMENT

Localized stages

Only about 20% of patients with MPM are candidates for potentially curative treatment.

Surgery is the first-line option for localized disease. The combined use of surgery, chemotherapy, and radiotherapy has not been established as first-line treatment due to conflicting data from the main studies conducted to date.

Radiotherapy is mainly used as adjuvant or neoadjuvant therapy in MPM. Radiotherapy with curative intent has limited applications in unresectable disease because of the challenges associated with treating large volumes with doses of > 60 Gy without damaging adjacent organs[55,56]. Radiotherapy alone is therefore mostly used for palliative purposes in MPM.

Surgery for staging and palliative purposes: The MesoVATS randomized clinical trial, which compared VAT-partial pleurectomy and standard talc pleurodesis in 196

Table 3 Eighth edition of the tumor, node, metastasis classification for mesothelioma

T	Primary tumor	N	Regional lymph nodes
Tx	Primary tumor cannot be assessed	Nx	Regional lymph nodes cannot be assessed
T0	No evidence of primary tumor	N0	No regional lymphnodemetastases
T1	Tumor limited to the ipsilateral parietal pleura with or without involvement of visceral pleuralmediastinal pleuradiaphragmatic pleura	N1	Metastases in the ipsilateral bronchopulmonary, hilar or mediastinal (including the internal mammary, peridiaphragmatic, pericardial fat pad or intercostal lymph nodes) lymph node)
T2	Tumor involving each of the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic and visceral pleura) with at least one of the following features: Involvement of diaphragmatic muscle. Extension of tumor from visceral pleura into the underlying pulmonary parenchyma	N2	Metastases in the contralateral mediastinal, ipsilateral or contralateral supraclavicular lymph nodes
T3	Locally advanced but potentially resectable tumor. Tumor involving all of the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic, visceral pleura) with at least one of the following features: Involvement of endothoracic fascia; Extension into the mediastinal fat; Non-transmural involvement of the pericardium; Solitary, completely resectable focus of Tumor extending into the soft tissues of the chest wall	M	Distant metastasis
T4	Locally advanced technically unresectable tumor. Tumor involving all of the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic and visceral pleura) with at least one of the following features: Diffuse extension or multifocal masses of tumor in the chest wall, with or without associated rib destruction; Direct transdiaphragmatic extension of tumor to peritoneum; Direct extension of tumor to the contralateral pleura; Direct extension of tumor to mediastinal organs; Tumor extending through to the internal surface of the pericardium with or without pericardial effusion, or tumor involving the myocardium	M0	No distant metastasis
		M1	Distant metastasis present

Table 4 Eighth edition of the tumor lymph nodes metastasis classification for mesothelioma

	T	N	M
Stage IA	T1	N0	M0
Stage IB	T2-T3	N0	M0
Stage II	T1-T2	N1	M0
Stage IIIA	T3	N1	M0
Stage IIIB	T1-T3	N2	M0
	T4	Any N	
Stage IV	Any T	Any N	M1

patients, found no significant differences in OS between the groups[57]. VAT-partial pleurectomy, however, was associated with better pleural effusion control and quality of life at 6 and 12 mo[57].

Surgery with radical intent-trimodality therapy: The goal of surgery with radical intent in MPM is to achieve macroscopic resection with maximal cytoreduction. Techniques include extrapleural pneumonectomy (EPP), pleurectomy/decortication (P/D), and extended P/D. Recommendations for the uniform definitions of these techniques were established by the IASLC Staging Committee and the International Mesothelioma Interest Group[58].

Trimodality therapy, which is the combination of chemotherapy, surgery, and radiotherapy, can increase survival in patients with resectable MPM. Nonetheless, over 50% of patients are unable to complete treatment due to toxicity and/or previous comorbidities (American Society of Anesthesiologists score > 3, forced expiratory volume in 1 s < 70%, and/or smoking)[59].

Multivariate analyses of retrospective data have shown that EPP is associated with slightly higher operative mortality and lower OS than P/D. Such comparisons, however, are prone to bias as choice of technique by a given institution depends on its experience[60,61]. Until further clinical evidence becomes available, both EPP and P/D can be considered for surgery with radical intent. Choice of technique should be decided by an expert committee on a case-by-case basis.

Studies of curative-intent trimodality therapy have evaluated different regimens. Induction chemotherapy has been shown to increase the rate of complete resections in early-stage MPM.

A multicenter Swiss trial that analyzed the effects of radiotherapy after neoadjuvant chemotherapy and EPP in patients with resectable MPM reported an encouraging median survival of 23 mo[62].

The European Organisation for Research and Treatment of Cancer investigated the feasibility of trimodality therapy with EPP in a phase II trial (European Organisation for Research and Treatment of Cancer 08031)[63]. The primary endpoint (treatment success defined as a patient who received the full treatment and was still alive 90 d after the end of treatment without progression or grade 3-4 toxicity) was not achieved. Another phase II trial investigating trimodality therapy in MPM that recruited 77 patients from nine institutions in the United States reported an operative mortality rate of 7% and a median OS of 16.8 mo[64].

Although trimodality therapy seemed feasible in selected patients[65], the Mesothelioma and Radical Surgery 1 randomized trial was designed to compare induction chemotherapy alone with induction chemotherapy plus EPP. It did not, however, achieve the planned sample size. Just 50 patients (45% of those recruited) were randomly assigned to either EPP or no EPP after chemotherapy, and just 16 of those in the EPP arm completed treatment. The mortality rate in this group was 18.8% [66]. The results of the subsequent trial, Mesothelioma and Radical Surgery 2, evaluating chemotherapy alone and chemotherapy followed by P/D are eagerly awaited.

The authors of a systematic review concluded that trimodality therapy can benefit selected patients in experienced centers[67].

One of the most important papers on trimodal therapy is the phase III trial by Trovo *et al*[68] published in 2020. In this trial, 108 patients with non-metastatic MPM, after non radical lung-sparing surgery and chemotherapy, were randomly assigned to receive hemithoracic radiation therapy (50 Gy in 25 fractions, gross residual disease received a boost of 60 Gy) or palliative radiation therapy (doses between 20-30 Gy). The primary endpoint was OS. With a median follow-up of 14.6 mo, the 2-year OS rate was 58% in the hemithoracic radiation therapy arm *vs* 28% in the palliative radiation therapy arm (hazard ratio, 0.54). However, in the hemithoracic radiation therapy group, the acute toxicity grade ≥ 3 was 20%, and the late toxicity grade ≥ 3-4 was registered in 17 patients (31% of the initial sample).

Adjuvant and neoadjuvant radiotherapy: Relatively little has been published on the use of adjuvant and neoadjuvant radiotherapy in MPM.

The Surgery for Mesothelioma after Radiation Therapy trial is a phase I-II trial that analyzed the feasibility of a short course of neoadjuvant hemithoracic IMRT (25 Gy in five fractions over consecutive days with a concomitant 5 Gy boost to risk areas) followed by EPP in patients with resectable T1-3N0M0 MPM[69]. The results were promising, with a cumulative 3-year survival rate of 84% in patients with epithelioid subtypes.

In 2016, de Perrot *et al*[70] published the results of a study of 62 patients treated with the Surgery for Mesothelioma after Radiation Therapy protocol. They reported a median survival of 36 mo. Respective median OS and disease-free survival (DFS) were 51 and 47 mo for patients with epithelioid-subtype tumors *vs* 10 and 8 mo for those with biphasic subtypes ($P = 0.001$).

Based on the results of the above two studies, neoadjuvant radiotherapy could be considered for patients with potentially resectable (T1-3N0M0) epithelioid subtype. Further randomized trials are needed.

The earliest studies of adjuvant radiotherapy in MPM reported high rates of serious adverse events. Pulmonary toxicity was particularly common after decortication.

The SAKK trial randomized 54 patients to observation or adjuvant radiotherapy after EPP but was terminated prematurely due to insufficient recruitment[71].

Nelson *et al*[72], using data from the United States National Cancer Database, analyzed the role of adjuvant radiotherapy in MPM patients treated between 2004 and 2013. Of the 2846 patients who underwent surgery, 213 (7%) received adjuvant radiotherapy, which was associated with better survival in patients with stage I-II disease ($P = 0.024$) but not in those with stage III ($P = 0.890$) or IV ($P = 0.183$) disease.

Prophylactic radiotherapy: The role of prophylactic radiotherapy in MPM is controversial, as the results of more recent randomized clinical trials contradict findings from older studies and small trials from the pre-chemotherapy era.

Two randomized trials comparing immediate radiotherapy to the tract (21 Gy/3 fractions) and observation only concluded that radiotherapy did not reduce the incidence of tumor seeding[73,74]. Similarly, the trial by Clive *et al*[75] found no differences between immediate and deferred prophylactic radiotherapy, contrasting with findings by Boutin *et al*[76] in 1995 indicating that early radiotherapy prevented malignant seeding.

Prophylactic radiotherapy is not recommended in clinical practice. Where possible, patients should be included in clinical trials such as prophylactic irradiation of tracts (NCT01604005).

Unresectable locoregional or metastatic disease

Chemotherapy is the treatment of choice for unresectable locoregional or metastatic MPM. It has been demonstrated to improve both survival and quality of life[77]. Nonetheless, palliative care is the only option in certain patients due to age, comorbidities, or poor general health.

The main goal of palliative radiotherapy is to alleviate pain caused by tumor invasion of the thoracic structures[78].

The evidence supporting radiotherapy to the chest for pain relief is based on a systematic review of retrospective series and small phase II trials[79]. The authors, however, were unable to draw any useful conclusions about the benefits of palliative radiotherapy in MPM or about optimal dose fractionation or target volumes due to the use of highly heterogeneous protocols and the fact that most of the techniques employed are now obsolete. The best evidence to date is from the multicenter phase II SYSTEMS-1 trial, which used modern radiotherapy techniques[80]. The trial included 30 patients who received 20 Gy (five fractions of 4 Gy/d). Pain, treatment response, and quality of life were evaluated using standardized questionnaires at weeks 1, 5, and 12 post-treatment. The results showed an improvement in pain in 47% of patients. No significant effects were observed for the other quality-of-life variables.

The encouraging results of SYSTEMS-1 led the researchers to design a second trial, SYSTEMS-2[81], to compare the effects of an escalated dose (36 Gy in six fractions for 2 wk) and a standard dose (20 Gy in five fractions for 1 wk) delivered only to the tumor responsible for the pain (hemithoracic radiation was not contemplated). The planned sample size is 112 patients and patients will be treated with IMRT or, where not available, three-dimensional (3D) radiotherapy. Radiotherapy quality, treatment responses, and associated toxicity will be evaluated using predefined protocols. No results have been reported yet.

Systemic therapy

First-line: The goal of first-line systemic therapy is to improve survival in patients with unresectable MPM. Superior survival outcomes have been reported for cisplatin plus pemetrexed or raltitrexed compared with cisplatin alone in phase III trials[77,82]. Carboplatin is an acceptable alternative, especially in older patients[83,84]. A number of clinical trials have investigated the effects of adding other agents to standard treatment. The phase III MAPS trial[85] showed the benefits of adding bevacizumab to the cisplatin-pemetrexed doublet, but this protocol has not been incorporated into general practice as it did not receive authorization from the United States Food and Drug Administration or the European Medicines Agency.

Maintenance: Continuation and switch maintenance chemotherapy with pemetrexed have changed clinical practice for non-small cell lung cancer. Their efficacy has not yet been demonstrated in MPM, but it is being investigated in the ongoing COMMAND and NVALT-19 trials.

Second-line: There is no current second-line standard-of-care systemic therapy for MPM, although a trend towards improved survival has been observed for vinorelbine [86,87], supporting findings for this drug as first-line therapy in the MSO1 trial[88].

Immunotherapy: The effect of tremelimumab on survival in patients with unresectable MPM is being evaluated in a phase III randomized trial (NCT01843374)[89]. PDL1 has also emerged as a promising target for immunotherapy, as it is significantly expressed by MPM and sarcomatoid mesothelioma in particular. In the absence of second-or further-line standard of care, patients should be encouraged to enroll in clinical trials where possible.

TARGET VOLUMES, ORGANS AT RISK, AND DOSE CONSTRAINTS

Defining target volumes for radiotherapy in MPM is challenging and requires thorough knowledge of the anatomy of the thorax and diaphragm and at times close collaboration with the thoracic surgeon.

Candidates for radiotherapy should have adequate performance status [electrocorticogram (ECOG) score ≤ 1] and respiratory function (forced expiratory volume in 1 s > 80%).

Immobilization techniques

Patients should be placed in a supine position with their arms raised over their head using a patient-specific immobilization device, such as an alpha cradle and headrest. Radiopaque fiducial markers and/or boluses should be placed in the region of the surgical incision and drainage areas.

CT images with a slice thickness of 2.5-3 mm should be acquired from 5 mm above the lung apex to the lower margin of the L3 vertebra or the anterior superior iliac spine, to include both kidneys.

Four-dimensional CT scans are recommended to determine the position of the diaphragm during respiratory movements. PET-CT may be necessary for postoperative restaging, particularly in patients with positive margins.

In patients with residual macroscopic disease, MRI with T1-and T2-weighted sequences, fat suppression, and diffusion-weighted imaging can be useful for determining macroscopic tumor volume[90]. Accuracy of target volume delineation can be improved by the intraoperative placement of fiducial markers at the level of the lower insertion of the diaphragm (or reconstructed diaphragm in cases of resection) and the anterior medial pleural reflection. It is also helpful to place these markers in any areas where the thoracic surgeon deems that complete resection may not be possible.

Target volumes

Clinical target volume (CTV): Volume encompassing entire pleura and ipsilateral chest wall as well as any sites at risk of residual disease (Figure 1).

Superior border: Above the first rib or 5 mm above the superior chest incision, whichever is higher.

Inferior border: Should encompass the entire diaphragmatic dome and diaphragmatic insertion as far as the emergence of the psoas muscle.

Anterior, posterior, and lateral borders: Should encompass the full thickness of the chest wall, ribs, intercostal muscles, lateral border of the sternum, costovertebral joints, lateral border of the vertebral bodies, costomediastinal and costodiaphragmatic recesses, and diaphragmatic crus. Anteromedially: Should encompass the anterior pericardium and the anterior medial pleural reflection. A 5-mm expansion margin should be used on the outer part of the chest wall, including skin in surgical incision and drainage areas. A 1-mm expansion margin should be used on the inner part of the chest wall in patients who have undergone lung-sparing surgery, bearing in mind that an additional 5 mm will be added for the planning target volume (PTV).

Medial border: Should encompass the mediastinal pleura and ipsilateral hilum. The inclusion of mediastinal lymph nodes is controversial. The proportion of positive mediastinal nodes ranges from 35%–41% depending on the surgical series[91,92], hence the recommendation by some groups to include these nodes in the radiation field[91,93-95].Although this positivity rate is high, most recurrences observed in cases where only involved mediastinal nodes were included occurred in the contralateral hemithorax (38%) or the abdomen (33%). Just 5% of local recurrences were classified as in-field failures[92]. Most groups therefore recommend only including mediastinal lymph nodes if they are positive[63,70,96-100].

PTV: Generally corresponds to the CTV with an isotropic expansion margin of 5 mm. It may, however, vary depending on the immobilization and/or image-control techniques used.

Radiation dose

The recommended radiation dose is 50–54 Gy in daily fractions of 1.8–2 Gy. Patients with residual disease will require a boost to at least 60 Gy. Organs at risk (OAR) dose constraints must be met at all times (Table 5).

OAR

OARs must be contoured according to the radiation therapy oncology group atlas[101]

Table 5 Dose constraints in organs at risk

Organs at risk	Dose constraints
Lungs-GTV	V20 < 37%, Mean dose < 20 Gy
Contralateral lung-PTV	V20 < 20%, V5 < 17%, Mean dose < 8 Gy
Ipsilateral lung	V40 < 67%, Mean dose < 36 Gy
Heart defined as pericardial sac	Right mesothelioma V40 < 25%; Left mesothelioma V40 < 35%
Brachial plexus	Maximum dose < 65 Gy
Esophagus	V55 Gy < 30%; Mean dose < 34 Gy
Stomach minus including PTV	Mean dose < 30 Gy
Bowel	Maximum dose < maximum PTV < 55 Gy; D5 cc < 50 Gy
Spinal cord	Maximum dose < 50 Gy
Liver minus GTV	V30 < 45%; Mean dose < 30 Gy
Kidneys evaluated separately	V18 < 33% (or V18 < 50%, if cannot be achieved at ≤ 33%)
Ipsilateral kidney; Contralateral kidney	V25 < 40%; V10 < 10%

GTV: Gross tumor volume; PTV: Planning treatment volume; V: Percent volume of organ permitted to receive specified dose.

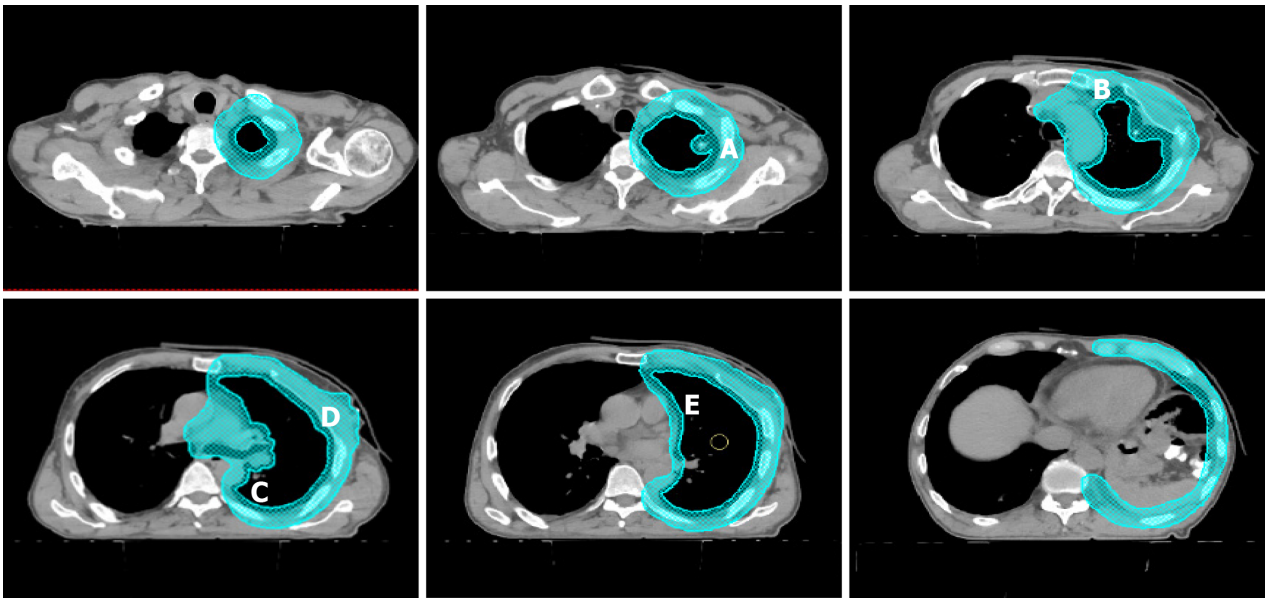


Figure 1 Clinical target volume: Volume encompassing entire pleura and ipsilateral chest wall as well as any sites at risk of residual disease. A: Chest wall and ribs; B: Lateral border of sternum; C: Costovertebral joints and lateral edge of vertebra; D: Surgical incision area; E: Anterior pericardium.

(Table 6).

Treatment planning

IMRT techniques are recommended for treatment planning. The PTV must be covered by at least 95% of the prescribed dose (Figure 2).

RADIOTHERAPY TECHNIQUES

Choice of radiotherapy technique depends on the clinical context, treatment goal (curative or palliative intent), planned dose, and location of the target volume and OARs.

Table 6 Dose constraints in organs at risk in mesothelioma radiotherapy treatment

Structure	Heart	Contralateral lung	Ipsilateral lung	Esophagus	Spinal cord
Dose constraints	V40: 0 (< 35%)	V20: 1.5 (< 20%); Mean dose 7 Gy (< 8)	V40: 57 (< 67%); Mean dose: 35 Gy (< 36 Gy)	V55: 0 (< 30%); Mean dose 26 (< 34 Gy)	Maximum dose: 43.7 (< 50 Gy)

In parentheses recommended limit values in relation to those reflected in Table 5 and those recommended by Gómez *et al*[116].

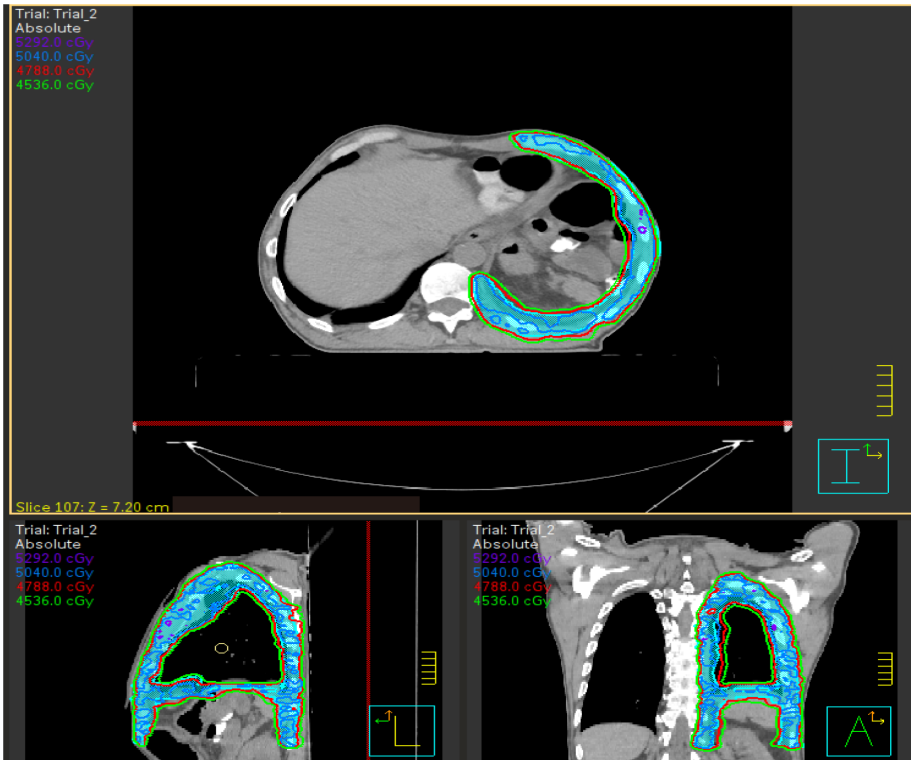


Figure 2 Volumetric modulated arc therapy with three complete arcs. Isodose: blue (5040 cGy-100%), red (4788 cGy-95%), purple (5292 cGy-105%).

Conventional two-dimensional or 3D techniques are suitable for most treatments with palliative intent. The doses are normally low (20 Gy in five fractions or 30 Gy in 10 fractions), and long-term toxicity is less of a concern given the poor prognosis[80].

Higher doses are needed when the goal is to achieve good long-term local control. More highly conformal techniques are recommended to achieve this goal with as little toxicity as possible.

Many studies of radiotherapy in MPM have used conventional postoperative two-dimensional hemothoracic radiotherapy with parallel opposing photon fields, with or without an electron boost. This technique results in good sparing of the contralateral lung but is associated with significant treatment inhomogeneities linked to a high risk of local failure and/or toxicity[102]. Technological advances brought with them a shift towards an increasing use of 3D conformal radiotherapy techniques, which enable improved definition of target volumes. Even more advanced techniques emerged, offering superior conformality, precision, image-guided control, and dose distribution [IMRT, volumetric modulated arc therapy (VMAT), helical tomotherapy (HT), and proton therapy[103]].

A typical clinical case of modern postoperative RT after P/D is showed in Appendix 1.

IMRT after EPP

IMRT and image-guided radiotherapy (IGRT) allow for the use of more highly conformal treatments, enabling thus the delivery of potentially curative doses to complex target volumes while maintaining doses to surrounding healthy tissue at an acceptable level[104-106].

The potential of IMRT as adjuvant therapy has been evaluated in several trials, the earliest of which demonstrated the feasibility of hemithoracic IMRT following EPP. Institutional studies of modern radiotherapy techniques have reported 2-year locoregional control rates ranging from 40%–71% and 2-year OS rates of 18%–57%[95,107-110].

In a study conducted at the MD Anderson Cancer Center (MDACC), EPP followed by IMRT was associated with a locoregional failure rate of just 13%[111]. Buduhan *et al*[112] reported that IMRT was associated with lower local recurrence rates than conventional radiotherapy in patients treated with induction chemotherapy and EPP.

Better local control rates were also observed in another two prospective phase II trials. In the first, Krug *et al*[64], studied a series of patients treated with EPP and adjuvant IMRT (54 Gy in 30 fractions) and reported a local failure rate of 20.4% and a median survival of 29.1 mo. In the second phase II trial, Van Schil *et al*[63] reported a locoregional recurrence rate of just 16.2% and a median survival of 33 mo in patients treated with trimodality therapy comprising postoperative radiotherapy at a dose of 54 Gy in 30 fractions.

Despite the excellent results reported in a number of studies[113], IMRT following EPP has been associated with severe pulmonary toxicity[56,111-114]. Researchers at the Dana-Faber Cancer Institute also reported high mortality rates attributed to IMRT [115]. The patients had been treated with IMRT after EPP and adjuvant chemotherapy and had received a dose of 54 Gy and a boost up to 60 Gy in high-risk areas (positive margins or areas with residual disease). Six of the 13 patients (46%) died after receiving IMRT. In a retrospective review of 63 patients treated with either 3D radiotherapy or IMRT, Rice *et al*[56] found a 6-mo mortality rate of 37% (23 patients). Of these 23 patients, six died of a fatal pulmonary event. Five (5.8%) of the patients in a study performed by Gómez *et al*[109] at the MDACC experienced grade 5 pulmonary toxicity.

Severe toxicity in the above studies was associated with neoadjuvant chemotherapy and several dosimetric parameters, namely V20 (contralateral lung volume receiving 20 Gy), V5, V10, and mean lung dose[111,115]. The recommendation thus was to restrict these doses for OARs.

The higher number of radiation beams or fields used in treatments plans for IMRT, VMAT, and HT means that the volume of tissue receiving low doses (5–10 Gy) may be larger, and this could directly affect toxicity. It is important to keep the above dosimetric parameters as low as possible for the contralateral lung. No specific thresholds have been established, but patients who experienced severe pulmonary toxicity in the above studies had mean lung dose > 8.5 Gy, V5 > 80%, V10 > 55% and V20 > 7%. The current recommendation thus is not to exceed these levels for any of the four parameters. V20 was the only independent dosimetric predictor of grade 5 pneumonitis or disease-specific death[111]. Dose constraints to the lung have become increasingly strict as our understanding of IMRT has improved. The result has been a reduction in the incidence of grade 3 or higher pneumonitis (< 10% in most series) and low rates of grade 3 or higher esophagitis, asthenia, and dermatitis[116,117].

Patel *et al*[117] found that greater experience with IMRT planning in patients with MPM following EPP was associated with statistically significant better coverage of target volumes and a significant decrease in the dose to the contralateral lung, with relatively low lung toxicity rates (13%).

Researchers from the University Hospital of Heidelberg analyzed IMRT delivered using the step-and-shoot technique or HT[110]. The median dose administered was 48–54 Gy, and there were no grade 4 or higher toxicities. Median OS was 20.4 mo. SAKK 17/04, an international multicenter phase II randomized trial, is the largest trial conducted to date of patients with MPM and the first to evaluate the role of high-dose hemithoracic radiotherapy as part of a multimodality approach. Following treatment with neoadjuvant chemotherapy (cisplatin/pemetrexed) and EPP, patients were randomized to receive or not high-dose radiotherapy (45.5 Gy in 26 fractions and a simultaneous integrated boost up to 55.9 Gy) to the hemithorax, the thoracotomy scar, and the mediastinal lymph node stations. The trial was terminated early due to low accrual. The difference in locoregional recurrence-free survival between patients in the high-dose radiotherapy group and the non-radiotherapy group was not significant (9.4 *vs* 7.6 mo)[70].

In conclusion, despite the results of the SAKK 17/04 trial, increasing experience with IMRT delivery and planning has led to improved toxicity profiles and lower local recurrence rates (15%–35%)[109,110]. Hemothoracic radiotherapy continues thus to be the recommended standard of care following EPP for patients with operable stage I to III MPM. This indication features in several recent guidelines[27-29,118], which also recommend that this technique be performed in centers of excellence with experience

in this modality.

IMRT after P/D

There is an increasing trend to treat operable MPM using more conservative, less invasive surgical techniques, such as P/D, which is associated with lower morbidity and mortality than EPP. P/D, however, may be less effective at achieving adequate cytoreduction and carries a higher risk of local and distant recurrence. The use of adjuvant radiotherapy thus must be contemplated in such cases. The presence of an intact ipsilateral lung in addition to other OARs after P/D also poses a major challenge to radiation oncologists[28].

The earliest studies of conformal radiotherapy following P/D showed a high incidence of grade 3 or higher toxicities (mostly pulmonary) and low survival rates [107,119].

Gupta *et al*[119] reviewed the records of 123 patients from the Memorial Sloan-Kettering Cancer Center treated with P/D followed by hemithoracic radiotherapy (3D with or without brachytherapy) over a 30-year period. With a mean follow-up time of 11 mo, median survival was 13.5 mo. Actuarial OS at 1 year was 23% and the local recurrence rate was 56.1%. The authors concluded that the combination of P/D and adjuvant hemithoracic radiotherapy was not effective and advised against the use of conventional IMRT in this setting[119].

Local failure rates following the clinical implementation of IMRT after P/D vary considerably from one study to the next, with 2-year rates ranging from 40%–68%; there have also been reports of severe toxicities[28].

According to a recent systematic review of the safety and efficacy of IMRT following P/D by Patel *et al*[120], between 0% and 16% of patients develop grade 3 pneumonitis, while less than 1.5% develop grade 4 or 5 pneumonitis. The authors also reported a recurrence rate of 19%–60%, a mean DFS time of 12–16 mo, and OS of 19–28 mo.

The Memorial Sloan-Kettering Cancer Center has published some of the most significant findings for IMRT as adjuvant therapy for MPM patients treated with P/D. Rosenzweig *et al*[121] reviewed the results of 36 patients treated with hemithoracic IMRT at this center; 20 had undergone P/D, and 16 had biopsy only. The dose in all cases was 50.4 Gy (1.8 Gy/fraction). The authors reported a grade 3 or higher toxicity rate of ≥ 20% and a median survival of 26 mo. They concluded that adjuvant IMRT following P/D improved disease-free and OS with acceptable toxicity levels[121]. The same group analyzed data from an additional 67 patients treated with IMRT (28 after P/D)[122]. The respective 1- and 2-year actuarial failure rates were 28% and 40%, and most of the recurrences were distant.

The first prospective phase II study to evaluate the safety of adjuvant hemithoracic IMRT after chemotherapy and P/D concluded that the administration of 50.4 Gy in 28 sessions was feasible[100]. There were no cases of grade 4 or 5 pneumonitis; 29.6% of patients developed radiation pneumonitis, and the median disease-free and OS times were 12.4 and 23.7 mo, respectively. The disease recurred locally in 58% of patients. Most of these recurrences were at sites with prior macroscopic disease, highlighting the importance of complete macroscopic resection.

Shaikhet al[123] retrospectively reviewed the data of 209 patients treated with 3D radiotherapy or IMRT after P/D. In the P/D-IMRT group, 65% of the 78 patients received > 45 Gy. The 2-year local failure rate was high at 60%, and there was one case each of grade 4 and grade 5 pneumonitis[123].

In a study of 24 patients treated with P/D and IMRT at a dose of 45 Gy with or without an integrated boost up to 57.5–60 Gy at the MDACC, there were no cases of grade 4 or 5 pulmonary toxicities, the median OS was 28 mo, and the crude local failure rate was 42%[98].

Shaaban *et al*[124] analyzed data from the National Cancer Database for 286 patients with MPM treated with IMRT. The median survival in patients treated with P/D and IMRT (63% of total) was 19 mo, but the difference with those treated with P/D and 3D radiotherapy was not significant. No information was given on toxicities.

Differences in the characteristics of surgery, chemotherapy, and radiotherapy may explain the heterogeneous results observed in the different series.

In conclusion, the combination of P/D and IMRT achieves acceptable disease control and is associated with a low rate of severe toxicities at radiation doses of 45–54 Gy[100]. IMRT can therefore be considered as an adjuvant therapy to P/D in well-selected patients. Enrolment for a phase III trial (NRG-LU006) comparing P/D plus chemotherapy with or without adjuvant IMRT (or intensity-modulated proton therapy) is expected to begin shortly.

VMAT and helical tomotherapy

Refined IMRT techniques were followed by VMAT and HT, whose superior technical and dosimetric qualities may offer even better outcomes in the setting of MPM (Figures 3 and 4)

VMAT involves the delivery of 6–10-MV-photon beams in a rotating arc, thereby offering better dose conformality compared with IMRT in addition to shorter delivery times and increased safety. HT uses a linear accelerator incorporated into a CT unit. It is a dynamic, rotational technique (the table moves during treatment) that uses multiple "fan-like" beams conformed at 51 different angles using an automated multileaf collimator. The photons generated in the linear accelerator have an energy of 6 MV. Both VMAT and HT offer high precision, high conformality, and daily IGRT.

Comparative dosimetric studies have confirmed the theoretical advantage of VMAT and HT over IMRT[125-128].

Dumane *et al*[126] reported shorter treatment times for VMAT *vs* IMRT (8 *vs* 20 min) in addition to better sparing of OARs. Krayenbuehl *et al*[127], in turn, found that VMAT allowed for the use of lower lung doses and that two partial arcs yielded the best dosimetry.

Kimura *et al*[129] published the first study on the efficacy and safety of VMAT following EPP in MPM. They analyzed 15 patients treated with 54 Gy in 30 fractions and reported a 1-year local control rate of 55.7% and an OS rate of 43.1%. Patients with non-epithelioid MPM had worse local control rates. Three patients (20%) developed grade 3 pneumonitis.

Sterzing *et al*[128], in a dosimetric study comparing HT and step-and-shoot IMRT, found that the former offered better target coverage and homogeneity and also allowed for the delivery of a mean dose of less than 5 Gy to the contralateral lung. Thieke *et al*[110], by contrast, found no significant differences in dosimetry or clinical outcomes between HT and step-and-shoot IMRT.

Minatel *et al*[130] conducted a prospective study to evaluate the safety of HT in patients with MPM treated with P/D. In an initial analysis, they reported the results for 28 patients treated with 50 Gy in 25 fractions with a simultaneous integrated boost up to 60 Gy (2.4 Gy/fraction) delivered to fluorodeoxyglucose-avid areas observed by PET. There were five cases of grade 2 or 3 pneumonitis. The authors found that contralateral lung V5 was strongly correlated with the risk of pneumonitis and therefore restricted this parameter to 17% for the contralateral lung. In a subsequent analysis of 69 patients, they observed seven cases of grade 3 pneumonitis, one case of grade 5 pneumonitis, and good disease control (local failure rate, 19%; 2-year OS rate, 58%–65%).

Now that technological advances enable better dose distribution control, new studies have emerged to analyze the feasibility of escalating doses without increasing toxicity. Parisi *et al*[131] analyzed 36 patients treated with HT: 19 after P/D and 17 with unresectable disease who had undergone biopsy only. The median dose was 25 Gy (range 25–30 Gy) delivered in five fractions. The results were very promising. The median survival for a median follow-up of 37 mo was 22 mo, and there were three cases of grade 3 pneumonitis and no cases of grade 4 or 5 pneumonitis.

In a study by Fodor *et al*[132], 51 patients were treated with hypofractionated HT at a dose of 56 Gy and most of them received a concomitant boost up to 62.5 Gy. There were no grade 3 pulmonary toxicities in the group that did not receive a boost. In the group that did receive the boost, however, there were two cases of grade 5 toxicity and 11 cases of grade 3 toxicity. Median OS was 26 mo.

Pehlivan *et al*[133] compared VMAT and HT in patients with unresectable MPM. They concluded that both techniques offered efficient target volume coverage while maintaining OAR doses under established limits, although HT was associated with better mean and maximum doses and a significantly more homogeneous dose distribution ($P < 0.001$). The main drawback of HT was its longer treatment time (7.4 *vs* 2.5 min/fraction, $P < 0.001$).

In conclusion, although more studies and clinical trials are needed, both VMAT and HT can improve local control in MPM and also have an acceptable toxicity profile. VMAT and HT should be performed in experienced centers.

Stereotactic body radiotherapy

High local recurrence is a major problem in MPM and could perhaps be improved through a more radical strategy targeting pleural and regional recurrences.

SBRT involves the delivery of high-dose radiotherapy in just a few sessions. It is a high-precision, highly conformal technique that uses a steep dose gradient. The goal is to deliver a highly targeted ablative dose to the tumor.

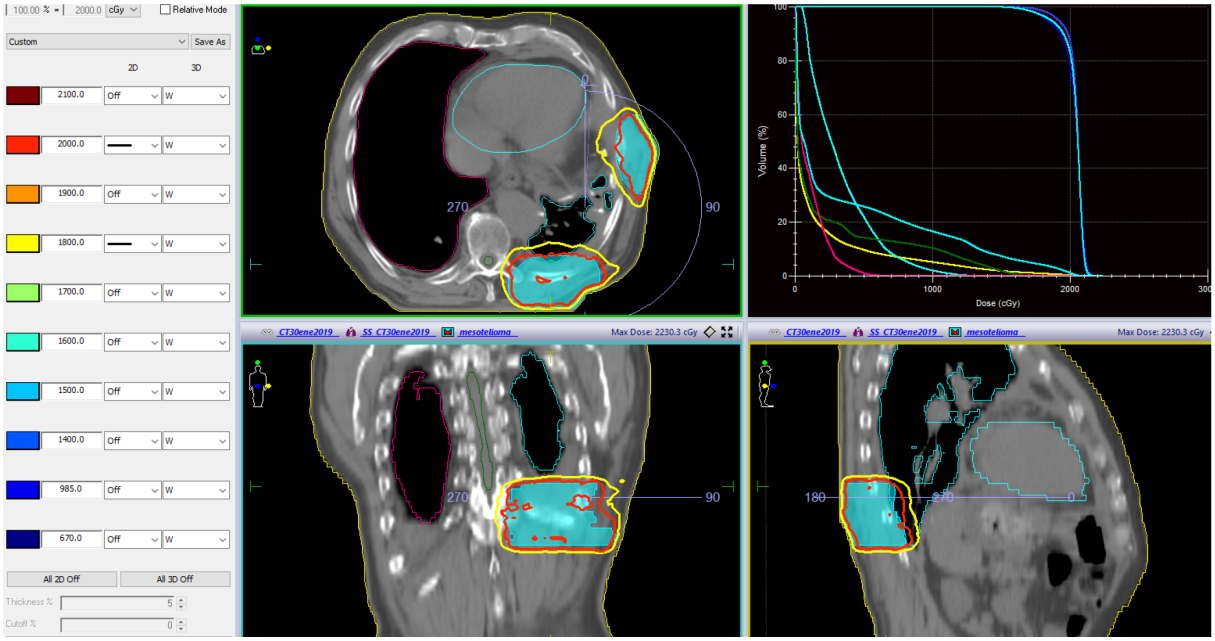


Figure 3 Patient with left malignant pleural mesothelioma treated with volumetric modulated arc therapy with palliative intent.

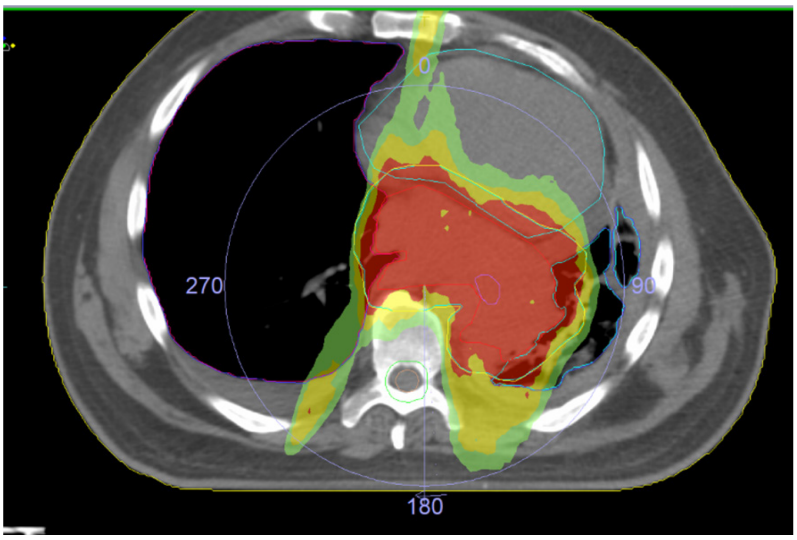


Figure 4 Patient with predominantly central malignant pleural mesothelioma treated with volumetric modulated arc therapy.

A Swiss group of researchers retrospectively evaluated the feasibility of SBRT for pleural recurrences in patients with oligoprogressive MPM[134]. They analyzed the data for 50 lesions in 21 patients who developed local recurrence after initial treatment of MPM. The patients received a median of five fractions (range 3–20) with a median dose of 5 Gy/fraction (range 2.5–12.5 Gy). The total median dose was 30 Gy (range 20–50 Gy). Median follow-up from diagnosis was 28 mo (range 7–152 mo). The local control rate achieved (calculated by lesion) was 73.5%. Median DFS was 6 mo (range 0–21 mo), and median OS from the first SBRT session was 29 mo (range 0–61 mo). Just one patient experienced grade 3 or higher toxicities. SBRT was well tolerated, even after multiple repetitions, and it was associated with high local control rates and promising OS.

SRBT may be a promising option for achieving local control and delaying the need for systemic therapy in selected patients with a low tumor burden.

Proton therapy

Radiotherapy can also be delivered using heavy particles with radiobiological properties, such as protons, carbon ions, neutrons, and alpha particles. These particles

deposit a high dose to the tumor but doses to adjacent tissues rapidly fall off (Bragg peak). The main challenge with proton therapy is that proton beams are sensitive to volume and changing densities (air cavities) in the tissues they cross and are also altered by respiratory and cardiac movements.

Few studies have analyzed the usefulness of proton therapy in MPM.

Krayenbuehl *et al*[135] compared dosimetric planning for IMRT and proton therapy in patients with MPM treated with radical surgery (EPP). They found that proton therapy provided better PTV dose coverage and homogeneity in relation to V95 (P = 0.04) and also delivered a lower dose to OARs (kidneys, contralateral lung, heart, spinal cord, liver). Dose distributions, however, were very sensitive to changing air cavities.

Researchers at the University of Pennsylvania evaluated the use of proton therapy in 16 patients with MPM after radical pleurectomy[136]. Doses were calculated as cobalt gray equivalents (CGE). The median dose was 51.75 CGE (range 50.0–75.0 CGE) in daily fractions of 2.0 CGE (range 1.8–2.5 CGE). With a median follow-up of over 5 mo, all the patients achieved local control. There were no cases of acute or late grade 3 or higher toxicities.

The same group performed the first prospective study of proton therapy with adjuvant or salvage intent (eight and two patients, respectively)[137]. The median dose was 55.0 CGE/1.8–2.0 CGE (range 50–75 CGE). The 2-year local control rate was 90% and median survival was 19.5 mo from treatment completion and 30.3 mo from diagnosis. None of the patients experienced grade 2 or higher acute or late toxicity.

Evidence on the use of proton therapy in patients with MPM following P/D is even scarcer. Badiyan *et al*[138] studied 10 such patients treated with a mean total dose of 54 Gy (range 50.0-60.0/1.8-2.4 Gy). Median follow-up was 6.5 mo, and the 6-mo local control and OS rates were 87.5% and 64.3%, respectively. The treatment was well tolerated, and just two patients experienced grade 3 pneumonitis.

In conclusion, proton therapy following either EPP or P/D may offer dosimetric advantages[138]. Although no randomized controlled trials have yet been conducted, proton therapy can be contemplated for selected patients at centers with experience, preferably within the context of a clinical trial[139].

Adaptative radiotherapy

Adaptive radiotherapy involves the creation of new plans during the course of treatment to adapt to changes in target volumes detected by IGRT. These changes may be the result of anatomic variations (changes in tumor volume, patient weight/waist circumference), organ movement, or variations in patient positioning.

Adaptive radiotherapy is a potentially useful tool for tumors such as MPM where it is difficult to meet OAR dose constraints due to the large treatment volumes required. There is, however, very little experience with this modality in the setting of MPM. Considering the lack of evidence and the few descriptions of its use in clinical practice, adaptive radiotherapy should be used with caution in MPM until more robust evidence becomes available.

RECURRENCE PATTERNS AND TREATMENT

Recurrence patterns

Patterns of recurrence in MPM vary widely between series. The most common pattern of recurrence is distant metastasis, which can occur alone or in association with local recurrence[107].

Distant metastasis was the most common pattern in the MDACC series of patients treated with EPP followed by hemithoracic IMRT. It occurred alone in 59% of cases and in association with local recurrence in 16%[109]. Just two patients (2.3%) developed local recurrence only. The most common site for locoregional recurrence was the ipsilateral hemithorax. Up to 57% of local recurrences were located in a previous radiotherapy field.

In the only prospective randomized controlled trial to analyze radiotherapy after EPP (SAKK 17/046), the recurrence rates were 5% for local recurrence only (within the PTV), 19% for synchronous distant and local recurrence, and 62% for distant metastasis only (outside the PTV)[92].

Based on data from small retrospective series with short follow-up times, recurrence patterns following P/D seem to differ somewhat from those seen in EPP, with higher rates of local recurrence (64% *vs* 31%) and lower rates of distant metastasis (36% *vs* 69%).

Treatment of recurrences

Very little has been published on the treatment of recurrence in MPM. Recurrence is a marker of poor prognosis (median survival of 3-mo following detection). In an analysis of 106 patients with recurrent disease, 74%were treated with salvage therapy (single modality in 86% of cases and a combination of treatments in 14%). The most common option was chemotherapy (71%) followed by radiotherapy (23%) and surgery (21%)[140]. The use of salvage therapy in these cases significantly increased DFS (10 *vs* 2 mo) ($P < 0.0005$).

Salvage surgery

The evidence on salvage surgery in MPM is controversial due to highly heterogeneous results and the use of small samples and different techniques. Salvage surgery can be contemplated on a case-by-case basis for patients who present exclusively with local recurrence amenable to surgery following trimodality therapy.

In a retrospective series of recurrence patterns and second-line treatments following multimodality therapy, 21% of patients presenting with local recurrence were treated with surgery, which was associated with an OS of 16 mo and significantly longer recurrence-free survival than either chemotherapy or radiotherapy ($P < 0.0005$)[140]. Resection of recurrent disease at the ipsilateral chest wall (9 patients) and extended chest-wall resections (4 patients) were safe and effective. Contralateral partial pleurectomy, by contrast (3 patients), was associated with high morbidity and mortality and is not recommended.

Salvage radiotherapy

Reradiation can be considered for the treatment of asymptomatic local recurrence. Doses and fractionation should be planned according to the site and extension of the tumor and the characteristics of the patient[28] (type of recommendation: Informal consensus, moderate level of recommendation).

Salvage radiotherapy has been administered using conventional (standard fractionation) or hypofractionated protocols, as monotherapy, and in combination with chemotherapy or after salvage surgery (positive margins). Heterogeneous outcomes have been reported.

The treatment of oligoprogressive disease is challenging, and an optimal approach has yet to be defined. Ablative treatment, however, may increase local recurrence–free survival. Based on results from other settings, SBRT may be a promising option for salvage radiotherapy in patients with oligoprogressive disease[134].

Salvage systemic therapy

Salvage chemotherapy provides poor results (median survival of 3–7 mo after recurrence)[29,141,142]. It can be considered in patients in good physical condition (ECOG ≤ 2) with unresectable local disease or distant metastasis. Patients who achieve a lasting response (> 6 mo) to induction chemotherapy with cisplatin/pemetrexed can be given the option of repeating this treatment or switching to vinorelbine, which is associated with better tolerance. Enrollment in a clinical trial should be considered given the limited efficacy of salvage chemotherapy.

Promising preliminary results for immunotherapy (pembrolizumab monotherapy or nivolumab +/- ipilimumab) have been observed in MPM since the first studies were published in 2009[29]. Two phase III trials (NCT02991482 and NCT03063450) are currently underway, but no results have been reported yet.

FOLLOW-UP

There are no clear evidence-based recommendations on post-treatment follow-up of patients with MPM. Many guidelines, including the European Society of Medical Oncology guidelines[30], recommend an individualized approach, determined by the patient's medical team. Signs or symptoms detected during follow-up, such as reduced respiratory function, chest pain, cough, anorexia, weight loss, and asthenia, can raise suspicion of disease progression. Nonetheless, some clinical guidelines recommend a CT scan every 3 to 6 mo after completion of active treatment[143]. The role of PET or MRI during the follow-up of patients with MPM is unclear. The development of targeted therapies and immunotherapy, which normally occurs within a clinical trial setting, will probably give rise to more objective follow-up strategies in the future [144].

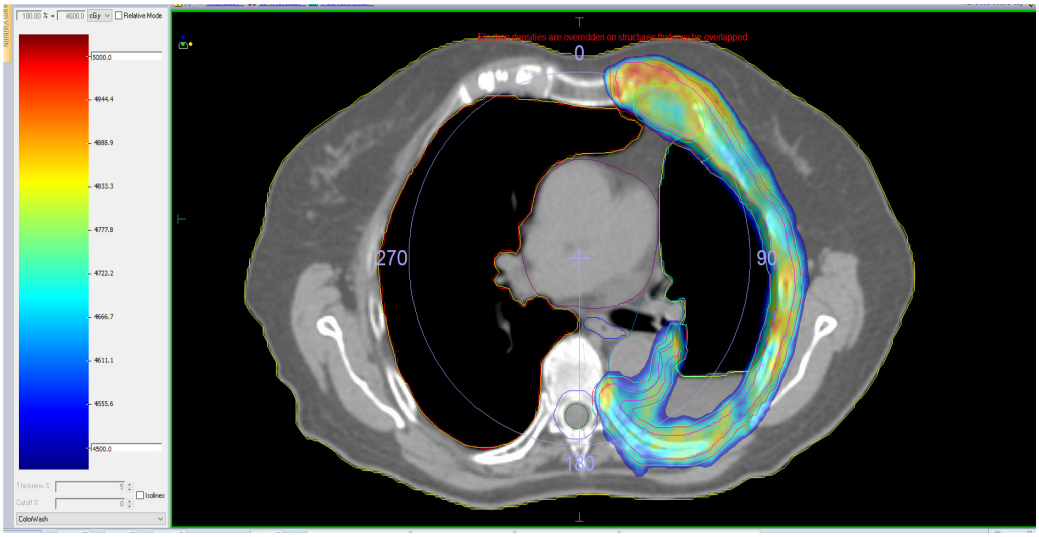


Figure 5 Dose distribution of radiotherapy treatment with volumetric modulated arc therapy. Note the adequate conformation to the clinical treatment volume, avoiding organs at risk.

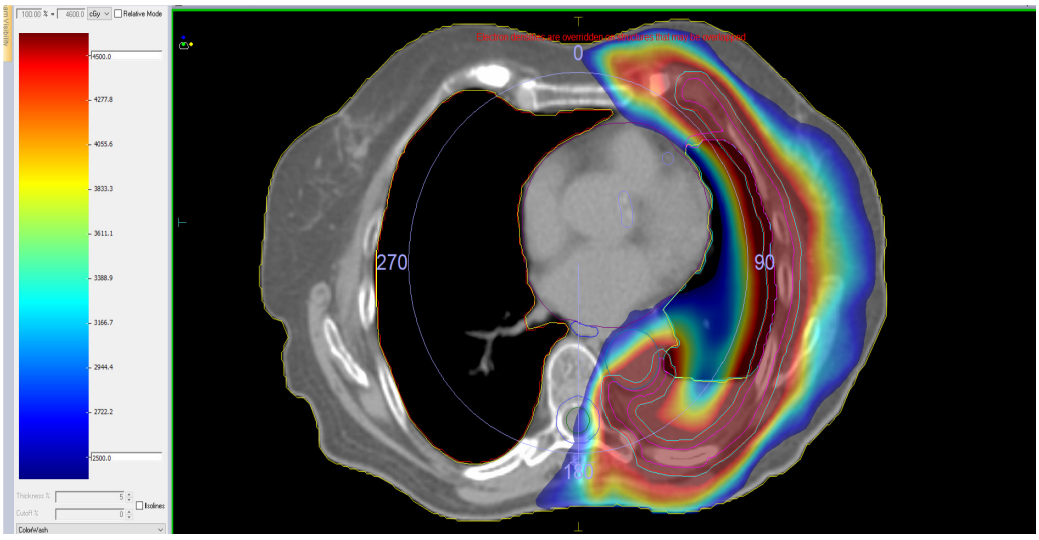


Figure 6 Low dose distribution of radiotherapy treatment with volumetric modulated arc therapy. Note the adequate conformation to the clinical treatment volume, avoiding organs at risk, specially the heart, esophagus, and contralateral lung.

APPENDIX 1: CLINICAL CASE

A 62-year-old male patient with a personal history of heavy ex-smoking of 20-30 cigarettes per day for 30 years until 2005. Occupational exposure to asbestos for 25 years. Melanoma in left lower limb treated with surgery.

Consultation in February 2020 for discrete increase of dyspnea without other significant associated symptoms. CT and PET-CT showed mild left pleural effusion and left pleural thickening predominantly in the apical area with suspected mesothelioma as the first option. The biopsy was positive for malignant mesothelioma.

The patient presented good respiratory functional data, so as it was a localized disease; treatment with neoadjuvant chemotherapy and subsequent surgery was considered by the tumor committee.

The patient presented a partial response to chemotherapy and non-radical lung-sparing surgery was proposed, obtaining a complete resection.

The pathological anatomy showed an epithelioid malignant mesothelioma, with disease predominating in the left latero-apical area, and two foci of disease in the caudal and medial area of the pleura.

Treatment was proposed with adjuvant RTE, with VMAT technique, with a dose of 46 Gy on total left hemipleura and a boost in areas of tumor persistence after

chemotherapy up to 60 Gy, with integrated boost technique, treatment in 23 sessions (Figures 5 and 6)

The established dosimetric criteria were met (Table 5), and the patient presented good tolerance to treatment, with the appearance of pleural effusion without associated symptoms during treatment.

CONCLUSION

MPM is a rare tumor that is very challenging to treat. Advances in surgery and radiotherapy, together with the development of new systemic therapies, have brought modest improvements in survival and local control and considerably lower toxicity than classic treatments Adequately powered, homogeneous clinical studies evaluating these advances are needed to provide objective evidence of improvements and clear evidence-based guidelines for the management of this disease.

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REVIEW

Roles of microRNAs in tumorigenesis and metastasis of esophageal squamous cell carcinoma

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Abstract

Esophageal squamous cell carcinoma (ESCC) is the major subtype of esophageal cancer that is prevalent in Eastern Asia. Despite recent advances in therapy, the outcome of ESCC patients is still dismal. MicroRNAs (miRNAs) are non-coding RNAs which can negatively modulate gene expression at the post-transcriptional level. The involvement and roles of miRNAs have become one of the hot topics of cancer research in recent years. In ESCC, genetic variations within miRNA coding genes were found to have distinct epidemiological significance in different populations. Dysregulated expression of several miRNAs was reported to be associated with therapeutic response. Functionally, miRNAs can act either in an oncogenic or a tumor-suppressive manner during tumorigenesis of ESCC by interrupting signaling pathways associated with cell proliferation, metabolism, cancer stemness, and resistance to chemo- or radiotherapy. Moreover, miRNAs modulate metastasis of ESCC by targeting genes that regulate cytoskeleton dynamics, extracellular matrix remodeling, epithelial-mesenchymal transition, and tumor microenvironment. Most importantly, mounting evidence suggests that inhibiting oncogenic miRNAs or restoring the loss of tumor-suppressive miRNAs has therapeutic potential in the treatment of ESCC. Here, we review and discuss recent studies on the significance, biological functions, and therapeutic potential of miRNAs in tumorigenesis and metastasis of ESCC.

Key Words: MicroRNAs; Dysregulation; Tumorigenesis; Metastasis; Therapeutic potential; Esophageal squamous cell carcinoma

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Core Tip: Esophageal squamous cell carcinoma (ESCC) is a deadly disease worldwide. Its poor prognosis is mainly due to the rapid tumor progression and high rate of

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invasion and metastasis. It is of great importance to understand the mechanisms underlying ESCC tumorigenesis and metastasis. Increasing studies confirmed the involvement of microRNAs (miRNAs) in cancer progression. Dysregulated miRNAs can serve as possible biomarkers for ESCC diagnosis or prognosis evaluation. Moreover, miRNAs function as small post-transcriptional regulators with notable therapeutic value. This review summarizes recent studies on the significance, biological functions, and clinical potential of miRNAs in tumorigenesis and metastasis of ESCC.

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INTRODUCTION

Esophageal cancer is one of the most aggressive cancers worldwide. According to 2018 global cancer statistics based on 185 countries, esophageal cancer is the seventh most common cancer and the sixth in terms of mortality globally[1]. The two major subtypes of esophageal cancer, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), differ in symptoms, geographic distribution, and etiology. ESCC is the most common subtype of esophageal cancer globally. It constitutes more than 80% of esophageal cancer cases worldwide[2-4]. In 2012, there were about 398000 new cases of ESCC, which was 7.6-fold higher than EAC cases[5]. Like many other cancer types, genetic alterations, uncontrollable proliferation, and escaping from cell death and immune-response are associated with the pathogenesis of ESCC. The high rates of local invasion and distant metastasis also contribute to the malignancy of ESCC. In fact, most patients have distant metastasis at initial diagnosis [6]. Delayed diagnosis and treatment lead to the poor prognosis. The 5-year survival rate of ESCC patients is only 20%-30%[7].

For decades, oncology studies mainly focused on dysfunction of protein-coding genes. It is only recently that the wide involvement of non-coding RNAs in cancer biology has been recognized. MicroRNAs (miRNAs) constitute one of the major families of non-coding transcripts. Typically, miRNAs are 20-24 ribonucleotides in length, and are transcribed from different genome locations, such as introns or junk DNA sequences between genes. MicroRNAs can bind to the 3'-untranslated region (UTR) of target mRNAs through imperfect base-pair complementation, and functionally promote target mRNA degradation or inhibit translation. The biogenesis of miRNAs is tightly controlled within normal cells, but mutations within miRNAs and dysregulated miRNA expression have been observed in many tumor types[8]. This highlights the possibility that miRNAs may be useful as diagnostic biomarkers. It has also been shown that miRNAs are involved in regulating multiple biological processes during cancer pathogenesis, which suggests that they may be exploited as therapeutic targets or tools[9]. In the last decades, increasing evidence supports that miRNAs have important roles in the pathophysiology of esophageal cancer[10,11]. In this review, we will summarize recent discoveries on the altered expression and functions of miRNAs in ESCC.

DYSREGULATION OF MICRORNAS IN ESCC

Genetic variations within miRNA sequence, as well as dysregulated level of miRNA, are frequently observed in multiple tumor types including ESCC[8]. Most of the genetic variations of miRNAs are due to the single nucleotide polymorphisms within miRNA coding genes (miR-SNPs)[12]. Studies in the last decades have demonstrated the epidemiological significance of miR-SNPs associated with susceptibility to ESCC in different populations. As a typical example, multiple studies reported that an SNP of miR-196a2, rs11614913 CC>TT, is associated with a reduced risk of ESCC in the Chinese Han population[13,14]. Another SNP of pro-miR-423, rs6505162 A>C, was found to be correlated with the incidence of ESCC in the Black population of South

Africa. The same study also emphasized that this allele is strongly associated with inhaling smoke from burning biomass fuel, which is a known environmental risk factor for ESCC[15]. Functionally, polymorphisms of miRNAs can affect the expression of mature miRNA oligos by changing the secondary structure and stability of pre-miRNA molecules[12,16]. Taking polymorphism of miR-196a2 as an example, the rs11614913 TT genotype is associated with reduced miR-196a expression, which weakens its oncogenic function[13]. Besides, SNPs located in miRNA binding sites within the 3'UTR of cancer-related genes are also regarded as a critical issue of miRNA dysregulation. For instance, SNP rs6573A>C in the 3'UTR of RAS-related protein (RAP1A) was found to eliminate miR-196a binding, which consequently increased RAP1A expression and promoted ESCC development[17]. Another example is that CT or TT genotype of rs2866943, which is an SNP of protein tyrosine phosphatase receptor type T (PTPRT) 3'UTR, was reported to be associated with disrupted miR-218-regulated PTPRT expression[18].

Compared with genetic alteration of miRNA by SNPs, dysregulated miRNA expression patterns in ESCC have attracted much more attention in recent years. Yang *et al*[19] performed miRNA profiling in 113 pairs of ESCC tumor and matched non-tumor tissues, and found that 39 miRNAs were dysregulated in ESCC by at least 2-fold, including 28 downregulated and 11 upregulated miRNAs. Within the list of downregulated miRNAs, some (*e.g.*, miR-133a and miR-133b) were also identified in other miRNA screening studies as possible tumor suppressors[20,21]. Dysregulation of several miRNAs was also reported to be associated with therapeutic response. For instance, miRNA profiling in ESCC tumor samples from patients receiving neoadjuvant radiochemotherapy showed that the expression of 12 miRNAs including 8 upregulated and 4 downregulated ones was altered in non-responders compared with responders[22]. In particular, high levels of miR-194 and miR-665, which were found in radiotherapy- or chemotherapy-resistant tumor samples, were correlated with a poor survival of ESCC patients, suggesting their potential as novel biomarkers for prognostic prediction[22]. Abnormal expression of miRNAs in ESCC may be due to many reasons. Genome instability is one of the underlying mechanisms. For example, Hu *et al*[23] performed global analysis of genome, mRNA, and miRNA status in 30 cases of ESCC, and demonstrated an association between bi-allelic loss in cancer genome and expression pattern change of 60 miRNAs. However, the authors also pointed out that bi-allelic loss in ESCC was infrequent, and that the relation between bi-allelic loss and miRNA dysregulation needs further validation[23]. Another piece of evidence is the location of some miRNA coding genes in the somatic copy-number alteration (SCNA) regions in cancer genome, such as miR-4448, miR-1224-3p, and miR-4707-5p in ESCC[24], which can lead to irregular mature miRNA expression. Moreover, several studies reported the correlation between dysregulated miRNA expression and abnormal epigenetic modulation in the promoter region. For example, promoter hypomethylation was found to enhance the expression of oncogenic miR-10-3p during ESCC development[25]. On the other hand, hypermethylation of CpG sites in promoter region was reported to downregulate the expression of tumor-suppressive miRNAs such as miR-375[26], miR-126[27], and miR-218[28,29] in ESCC. In addition, further studies demonstrated that miRNA expression is regulated by multiple upstream epigenetic factors, which is usually defined as "epigenetic-miRNA feedback loop"[30]. Taking miR-126 and miR-218 as examples, hypermethylation of their promoters is mediated by members of the DNA methyltransferase (DNMT) family, especially DNMT1 and DNMT3B[27,29]. Besides, epigenetic chromatin modifications also contribute to regulation of miRNA expression pattern. Koumangoye *et al*[31] reported that trimethylation of histone H3 lysine 27 (H3K27me3), which is a well-known heterochromatic modification, negatively regulated the expression of miR-31 in ESCC cells. In that study, the authors found that the SOX4/EZH2/HDAC3 co-repressor complex mediates hypermethylation of H3K27 around miR-31 promoter [31]. However, up to now, little is known about the regulatory function of other types of histone modifications on miRNA expression in ESCC. Further studies are needed to reveal the possible contribution of different histone modifications in modulating miRNA expression pattern in ESCC.

ROLES OF MICRORNAS IN TUMORIGENESIS OF ESCC

Beyond merely identifying alterations in miRNA expression patterns, researchers have become more focused on studying the functional significance of aberrantly expressed miRNAs. MiRNAs have been found to be involved in regulation of multiple biological

processes during ESCC development (Figure 1). According to the distinct roles, miRNAs can be broadly described as tumor-suppressive and oncogenic miRNAs. Generally speaking, tumor-suppressive miRNAs functionally impede tumor malignancy, and are frequently repressed in expression level in tumor cells. For example, miR-503 was reported to negatively regulate cyclin D1 and induce cell cycle arrest in G1/S phase in ESCC[32]. Similar cell proliferation-repressive miRNAs include miR-200c and miR-593*, which interfere with mitosis by targeting P21 and target polo-like kinase 1 (PLK1), respectively[33,34]. Some miRNAs also suppress development of ESCC by regulating metabolic processes, especially glucose metabolism. For instance, miR-375 exerts a metabolism-repressive role by directly inhibiting lactate dehydrogenase B, which is a key enzyme in post-glycolysis process and catalyzes pyruvate-lactate interconversion[35]. Similarly, rate-limiting enzymes in glycolysis, such as glucose-6-phosphate dehydrogenase (G6PD) and hexokinase 2 (HK2), were reported to be negatively regulated by miR-613[36] and miR-125/143 cluster in ESCC[37]. Besides, miRNAs can also exert tumor-suppressive function by inhibiting cancer stemness. For example, Li *et al*[38] found that miR-377 was frequently downregulated in ESCC tumor, and that ectopic miR-377 expression could inhibit sphere formation and tumorigenic potential of ESCC cells by directly inhibiting CD133, which is a well-known stemness biomarker in cancer. In addition, it was reported that miR-377 can target chromobox protein homolog 3 (CBX3) and contributes to maintenance of stem cell potential in ESCC[39], which further emphasized the importance of miR-377 as a critical tumor suppressor in ESCC.

Moreover, tumor-suppressive miRNAs can also promote the effect of chemoradiotherapies. A previous study by our group reported the tumor-suppressive role of miR-29c in reducing chemoresistance[40]. Overexpressing miR-29c in fluorouracil (5-FU)-resistant ESCC cell sublines increased their response to 5-FU treatment, shown as decreased cell viability and increased cell death, while miR-29c antagonist had opposite effect and could desensitize parental cells to 5-FU treatment. F-box only protein 31 (FBXO31), which has oncogenic function in ESCC[41] and lung cancer[42], was found to be one of the direct target genes of miR-29c that mediates its tumor-suppressive function. Further investigation found signal transducer and activator of transcription 5A (STAT5A) to be a transcription factor that can negatively regulate miR-29c expression in 5-FU-resistant cells[40]. This study highlighted the key role of the STAT5A/miR-29c/FBXO31 axis as a modulator of ESCC chemoresistance. By utilizing the same 5-FU-resistant cell model, Han *et al*[43] proved that miR-338-5p directly targets Id-1 (Inhibitor of DNA Binding 1) in ESCC cells to reverse chemoresistance. Interestingly, miR-338-5p was also able to attenuate cisplatin resistance and enhance radiotherapy efficiency by targeting focal adhesion protein kindlin-2 (also known as FERMT2)[44] and survivin[45], respectively. These studies suggest that miR-338-5p may be an essential tumor inhibitor.

On the other hand, onco-miRNAs are often upregulated in expression and functionally promote cancer progression by inhibiting tumor-suppressive proteins. One of the most well-studied oncogenic miRNAs in ESCC is miR-21. Several studies have reported that this miRNA is upregulated in the tumor[46-48], as well as in the serum or plasma of patients with ESCC[49-51]. Functionally, miR-21 was reported to promote cell growth and inhibit apoptosis in ESCC by activating the ERK/mitogen-activated protein kinase (MAPK) signaling cascade[52], or targeting genes including Fas ligand (FASL)[53], programmed cell death 4 (PDCD4)[54], phosphatase and tensin homolog (PTEN)[55-57], and RAS p21 protein activator 1 (RASA1)[58]. Further study also found miR-21 to play a role in maintaining cancer cell stemness in ESCC by upregulating stem cell markers such as Oct4 and Nanog and targeting TNF receptor-associated factor 4 (TRAF4)[59]. Oncogenic effects of miR-21 were also reported in breast cancer[60] and colorectal cancer[61], indicating its potential value as a pan-cancer therapeutic target. Onco-miRNAs can also interrupt cancer-related inflammation. For instance, miR-31 was found to be overexpressed in an orthotopic ESCC rat model promoted by Zn-deficiency, and functional tests revealed that miR-31 knockout abolished ESCC development and altered the expression profile of inflammation genes [62]. This effect was mediated by tumor suppressors egl-9 family hypoxia inducible factor 3 (EGLN3) and membrane bound o-acyltransferase domain containing 2 (MBOAT2)[62]. Actually, both oncogenic and tumor-suppressive functions of miR-31 were found in different cancer types[63], but knockout of miR-31 failed to show obvious genomic and metabolic instability in the rat esophagus, indicating that it might be of great value as a potential therapeutic target for ESCC[62].

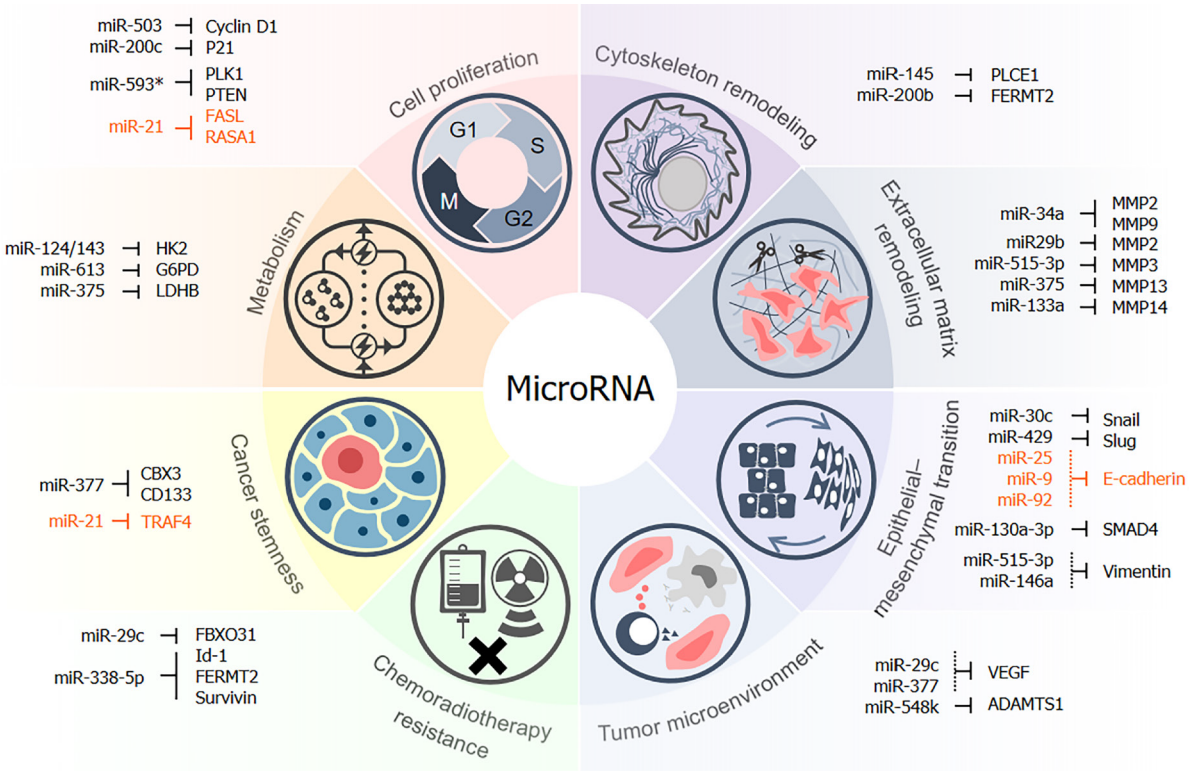


Figure 1 Multiple roles of miRNAs during development of esophageal squamous cell carcinoma. This figure summarizes the targets and regulatory functions of miRNAs in multiple biological processes during esophageal squamous cell carcinoma (ESCC) development. MicroRNAs inhibit or promote tumorigenesis and recurrence of ESCC by modulating cell proliferation, metabolism, cancer stemness, and resistance to chemo- or radiotherapy. They also regulate metastasis by targeting functional molecules involved in epithelial-mesenchymal transformation, remodeling of the cytoskeleton, extracellular matrix, and tumor microenvironment. Oncogenic miRNAs and target genes are marked in orange. PLCE1: Phospholipase C epsilon 1; VEGF: Vascular endothelial growth factor.

ROLES OF MICRORNAS IN METASTASIS OF ESCC

The high incidence of metastasis is a serious issue associated with poor prognosis of ESCC. In addition to investigating the involvement of protein-coding genes in modulating tumor metastasis, recent studies have provided much information on the regulatory roles of miRNAs (Figure 1, also summarized in Table 1). MiRNAs were reported to modulate motility of ESCC cells by regulating cytoskeleton dynamics. For instance, miR-145 was demonstrated to have a suppressive effect on metastasis of ESCC by targeting phospholipase C epsilon 1 (PLCE1)[64], which is a Ras protein associated effector that can functionally remodel actin cytoskeleton[65,66]. Another example is miR-200b, which was reported to target FERMT2[67]. Therefore, the miR-200b/FERMT2 regulatory axis can reduce cell invasiveness by modulating the structure of the cytoskeleton and blocking the formation of focal adhesion[67].

The invasiveness of cancer cells is largely dependent on remodeling of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs)[68]. Members of the MMP family are endopeptidases that catalyze the degradation of ECM components. In ESCC, highly expressed MMPs are often associated with strong malignancy and poor prognosis[69,70]. MiRNAs can modulate ESCC cell invasiveness by targeting MMPs. Yang *et al*[71] reported that a p53-downstream miRNA, miR-34a, suppressed ESCC cell migration and invasion by directly targeting and suppressing MMP2 and MMP9. Interestingly, miR-34a was also found to inhibit the expression of an upstream transcription factor of MMP2 and MMP9, namely, Yin Yang 1 (YY1), in ESCC cells, so that MMP2 and MMP9 levels were negatively regulated in ESCC[72]. Besides, MMP2 was also reported to be regulated by miR-29b in ESCC[73]. Qi *et al*[73] showed that the miR-29b/MMP2 axis inhibited cell invasion *in vitro*, as well as tumor growth in an animal model, indicating its involvement in both tumor development and metastasis. Other members of the MMP protein family, such as MMP3, MMP13, and MMP14, were found to be regulated by miR-515-3p[74] , miR-375[75], and miR-133a[76], respectively.

Apart from gain of cell motility and remodeling ECM by MMPs, epithelial-mesenchymal transition (EMT) is another important process during pre-metastasis stage. Generally speaking, EMT describes the loss of epithelial cell phenotype to

Table 1 Metastasis-regulating miRNAs in esophageal squamous cell carcinoma

MicroRNA	Validated target(s)	Effect	Ref.
miR-1	Notch2	Represses proliferation, migration, and invasion	[105]
miR-9	E-cadherin	Promotes metastasis	[91]
miRNA-10b-3p	TSGA10	Promotes cell growth and metastasis	[106]
miR-17/20a	TGFBR2	Represses migration and invasion	[107]
miR-21	TGFβ	Promotes TGFβ-induced EMT	[108]
	PCD4	Promotes cell growth and invasion	[54]
	PTEN	Promotes invasion	[57]
miR-25	E-cadherin	Promotes metastasis	[93]
miR-26a and miR-144	COX2	Repress proliferation and metastasis	[109]
miR-29b	MMP2	Represses proliferation and invasion	[73]
miR-29c	VEGF	Represses angiogenesis and metastasis	[95]
miR-30c	SNAI1	Represses proliferation, EMT and invasion	[86]
miR-34a	MMP2, MMP9, and FNDC3B	Represses migration and invasion	[71]
	CD44	Represses invasion and metastasis	[110]
	Yin Yang-1	Represses migration and invasion	[72]
miR-92a	E-cadherin	Promotes lymph node metastasis	[92]
miR-92b	ITGAV	Represses invasion and metastasis	[111]
miR-106b	PTEN	Promotes invasion and metastasis	[112]
	SMAD7	Represses EMT	[84]
miR-128-3p	ZEB1	Represses EMT and metastasis	[89]
miR-130a-5p	ZEB1	Represses EMT and metastasis	[113]
miR-130-3p	SMAD4	Represses EMT, migration, and invasion	[82]
miR-133a	MMP14, FSCN1	Represses cell invasion	[76]
miR-145	PLCE1	Represses proliferation and metastasis	[64]
miR-146a	Vimentin	Represses tumor invasion	[85]
miR-150	ZEB1	Represses EMT and metastasis	[90]
miR-200b	Kindlin-2	Represses focal adhesion formation and invasion	[67]
miR-218	BMI1	Represses proliferation and metastasis	[114]
miR-339-5p	TSPAN15	Represses metastasis	[115]
miR-375	SHOX2	Represses invasion and metastasis	[116]
	MMP13	Represses migration and invasion	[75]
miR-377	CD133 and VEGF	Represses tumor initiation and progression	[38]
miR-424-5p	SMAD7	Represses EMT, migration and invasion	[83]
miR-429	Slug	Represses migration and invasion	[87]
miR-515-3p	MMP3 and Vimentin	Represses invasion and metastasis	[74]
miR-548k	ADAMTS1	Promotes lymph node metastasis	[96]
miR-630	Slug	Represses invasion and metastasis	[117]
miR-644a	PITX2	Repressing aggressiveness and stem cell-like phenotype	[118]
miR-655	TGFBR2 and ZEB1	Represses EMT	[88]
miR-1290	SCAI	Promotes proliferation and metastasis	[119]
miR-4324	FAK	Represses EMT	[120]

miR-6775-3p	MAGE-A family proteins	Represses invasion and metastasis	[121]

TGF-β: Transforming growth factor β; PTEN: Phosphatase and tensin homolog; PLCE1: Phospholipase C epsilon 1; VEGF: Vascular endothelial growth factor.

assume a mesenchymal cell phenotype. It is considered to be a transitional process with a spectrum of stages, such as losing cell polarity and cell-cell junction, enhancing migratory property and invasiveness, and acquiring resistance to anoikis[77-79]. The EMT process is controlled by many growth factors, but the dominant inducer is considered to be transforming growth factor β (TGF-β)[80]. TGF-β stimulates Smad and MAPK signaling, thereby inducing a series of genetic events during EMT. Activation of SNAIL, ZEB, and TWIST transcription factor families is the initiation step. These transcription factors silence the gene expression of tight junction molecules of epithelial cells such as E-cadherin, tight junction protein 1 (TJP1), and zonula occludens 1 (ZO-1), and translationally activate the expression of mesenchymal marker molecules such as N-cadherin and vimentin[81]. Up to now, several miRNAs have been found to regulate the EMT process by directly targeting these related elements during metastasis of ESCC. For example, miR-130a-3p was reported to suppress EMT in ESCC by targeting SMAD4, which is one of the downstream molecules of the TGFβ signaling pathway[82]. Similarly, SMAD7 can be regulated by miR-424-5p[83] and miR-106b[84]. The miR-515-3p/vimentin regulatory axis can reverse EMT and negatively regulate ESCC metastasis[74]. Another miRNA that targets vimentin, namely, miR-146a, was also reported to suppress ESCC invasion by disrupting fibronectin membrane assembly[85]. Two members of the SNAIL transcription factors family, Snail and Slug, were found to be regulated by miR-30c[86] and miR-429[87], respectively. The transcription factor ZEB1 is negatively regulated in ESCC by miR-655 [88], miR-128-3p[89], and miR-150[90]. On the contrary, miR-25, miR-9, and miR-92 act as oncogenic miRNAs that can promote EMT and ESCC metastasis by targeting E-cadherin[91-93].

Metastasis is affected by biochemical and cellular components surrounding tumor cells, which constitute the tumor microenvironment (TME). MiRNAs are functionally involved in the interaction between ESCC cells and the TME. On the one hand, the expression pattern of miRNAs in ESCC cells can be modulated by the TME, especially under stressful conditions such as hypoxia[94]. On the other hand, miRNAs are also known to regulate the crosstalk between ESCC cells and surrounding cancer-associated cells. In particular, several studies have found that miRNAs modulate the expression or secretion of vascular endothelial growth factor (VEGF). For instance, one of the previous studies in our group reported that miR-29c could suppress VEGF expression in cancer-associated fibroblasts, and the expression of miR-29c was in turn regulated by insulin-like growth factor 2 (IGF2) secreted by ESCC cells, suggesting the existence of a IGF2/miR-29c/VEGF regulatory loop which can promote ESCC distant metastasis[95]. Another study performed by our group found that VEGF was also targeted and inhibited by miR-377 in ESCC cells, so that the miR-377/VEGF axis had a negative impact on ESCC metastasis by downregulating angiogenesis[38]. In addition, Zhang *et al*[96] demonstrated that miR-548k directly inhibited metalloproteinase ADAMTS1, which acted as a chaperone and blocked intrinsic VEGFC. In addition, high level of miR-548k in ESCC cells facilitated VEGFC secretion. As a consequence, the miR-548k/ADAMTS1/VEGFC axis promoted lymph node metastasis by activating VEGFR3 in lymphatic endothelial cells[96].

THERAPEUTIC POTENTIAL OF MICRORNAS IN ESCC

In view of increasing evidence proving that miRNAs have important regulatory roles in cancer, miRNAs are considered as a pool of ideal therapeutic targets for developing novel treatment strategies. To be specific, treatment with synthetic antagonists or inhibitors may specifically neutralize and block the endogenous activity of oncogenic miRNAs. This is proposed as "mRNA suppression therapy". As for tumor-suppressive miRNAs, "miRNA replacement therapy", which is defined as restoring the loss of tumor-suppressive miRNA and enhancing the post-transcriptional repression using miRNA mimicking oligonucleotides or agomirs, is appropriate[97,98]. As a type of gene therapy, miRNA-based therapy has numerous advantages. First, the small molecular weight of miRNA oligonucleotides[98] renders them easier to deliver *in vivo*

than plasmids or viral-based gene therapies. Second, miRNAs act as network regulators and can mediate silencing of multiple target genes or pathways simultaneously. This is believed to induce enhanced anti-cancer effects, compared to conventional therapies such as chemoradiotherapy and single-targeting inhibitors[97,99]. Third, miRNAs have lower toxicity compared with DNA or protein-based therapies because they are endogenously produced by cells[98,99]. Therefore, novel miRNA-based cancer therapies have become an exciting development in recent years.

Current studies on miRNAs in ESCC mostly focus on the identification of clinical correlation and novel targeting axes[100-102]. Less is known about the therapeutic potential of specific miRNAs in treatment. Isozaki *et al*[103] showed that subcutaneous injection of miR-375 suppressed ectopic ESCC tumor growth *in vivo*. Another miRNA, miR-27a, had similar tumor-suppressive effect on ESCC tumor growth *in vivo* after direct injection into implanted tumor[104]. Most recently, three miRNAs including miR-377, miR-29c, and miR-515-3p proved to have therapeutic potential through systemic delivery *via* intravenous injection in mouse models[38,40,74]. Specifically, systemically administered miR-377 was able to suppress the growth of subcutaneous ESCC tumor xenografts and lung metastasis in a mouse model[38]. Systemic delivery of miR-29c produced a synergistic effect in inhibiting tumor growth when combined with 5-FU treatment[40]. Oligonucleotide mimicking miR-515-3p inhibited lung metastasis of ESCC in nude mice[74].

CONCLUSION

Studies in recent years have underscored the salient involvement of miRNAs in cellular events and human disease. A number of dysregulated miRNAs have been identified in multiple cancer types and nominated as potential novel cancer biomarkers. We now have a better understanding of how miRNAs can simultaneously regulate multiple target genes, and thereby functionally behave as oncogenes or tumor suppressors during cancer development and progression. Most importantly, researchers have discovered the therapeutic potential of miRNAs as novel targets or agents. In summary, studies in ESCC confirmed that miRNAs form a complex regulatory network in modulating tumor progression and malignancy. However, related investigations on the clinical application of miRNAs are still scarce. Therefore, future in-depth research on regulatory mechanisms of miRNAs and further validation of their therapeutic efficacy will allow us to better exploit this ubiquitous class of small molecules.

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REVIEW

Liver transplantation in malignant disease

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Abstract

Liver transplantation for malignant disease has gained increasing attention as part of transplant oncology. Following the implementation of the Milan criteria, hepatocellular carcinoma (HCC) was the first generally accepted indication for transplantation in patients with cancer. Subsequently, more liberal criteria for HCC have been developed, and research on this topic is still ongoing. The evident success of liver transplantation for HCC has led to the attempt to extend its indication to other malignancies. Regarding perihilar cholangiocarcinoma, more and more evidence supports the use of liver transplantation, especially after neoadjuvant therapy. In addition, some data also show a benefit for selected patients with very early stage intrahepatic cholangiocarcinoma. Hepatic epithelioid hemangioendothelioma is a very rare but nonetheless established indication for liver transplantation in primary liver cancer. In contrast, patients with hepatic angiosarcoma are currently not considered to be optimal candidates. In secondary liver tumors, neuroendocrine cancer liver metastases are an accepted but comparability rare indication for liver transplantation. Recently, some evidence has been published supporting the use of liver transplantation even for colorectal liver metastases. This review summarizes the current evidence for liver transplantation for primary and secondary liver cancer.

Key Words: Liver transplantation; Hepatocellular carcinoma; Cholangiocellular carcinoma; Hepatic epithelioid hemangioendothelioma; Undifferentiated embryonal sarcoma of the liver; Colorectal cancer liver metastases; Neuroendocrine cancer liver metastases

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Core Tip: This review focuses on the role of liver transplantation in the treatment of primary and secondary liver cancer. Particularly, we summarize the selection criteria for hepatocellular carcinoma and the available evidence for liver transplantation in perihilar and intrahepatic cholangiocarcinoma. Very rare indications such as hepatic epithelioid hemangioendothelioma, fibrolamellar carcinoma, hepatic angiosarcoma and undifferentiated embryonal sarcoma of the liver are reviewed. In secondary liver cancer, neuroendocrine liver metastases constitute the only established indication so far, and the existing data is recapitulated. Regarding colorectal liver metastases, current novel evidence and ongoing studies are summarized.

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INTRODUCTION

Liver transplantation is increasingly recognized as the curative treatment option in various end-stage liver diseases. Over the last 50 years, progress in operative technique and perioperative management as well as increasing knowledge about patient selection and postoperative immunosuppression have led to significant improvement in peri- and postoperative outcomes. As a result, 1-year overall survival (OS) rates after liver transplantation nowadays range between 80% and more than 90%, while 5-year OS has been reported to be around 70%[1-3]. So far, a wide variety of indications for liver transplantation are generally accepted, including cirrhosis, acute liver failure, and metabolic disorders[4]. However, the initial attempts to employ liver transplantation as treatment for malignant diseases were disappointing because of early recurrence and disease progression. In 1996, Mazzaferro *et al*[5] introduced the so-called Milan criteria for patients suffering from hepatocellular carcinoma (HCC). The implementation of those strict criteria led to improvements of both OS and recurrence-free survival (RFS)[5], emphasizing the importance of careful patient selection as key factor when applying liver transplantation for malignant disease. Subsequently, promising results have been published regarding not only cholangiocarcinoma but also for neuroendocrine cancer liver metastases (NECLM). Recent evidence also suggests that colorectal liver metastases (CRLM) are a good indication for liver transplantation in certain situations. The latter shows that the meaning of liver transplantation in the field of oncology is constantly changing. To reflect its increasing importance, the term "transplant oncology" was established[6].

A breakthrough in solid organ transplantation was the development of immunosuppressive drugs to avoid organ rejection. Particularly, the implementation of the calcineurin inhibitor (CNI) cyclosporine reflects a cornerstone in immunosuppression. Subsequently, novel drugs such as the CNI tacrolimus[7], the antimetabolite mycophenolate mofetil[8], the monoclonal antibody basiliximab[9] and the mammalian target of rapamycin (mTOR) inhibitor everolimus[10] have been introduced into routine care after liver transplantation. Current regimes for early post-transplant immunosuppression are mainly based on a combination of two to three drugs, with CNI being the backbone. A major concern regarding the use of CNIs upon liver transplantation for malignant disease is their tumor-promoting effect, as suggested by experimental data[11,12], and also the risk of de-novo development of secondary malignancies. In contrast, mTOR inhibitors such as sirolimus and everolimus have shown antineoplastic efficacy in preclinical tumor models[13-15]. Hence, the obvious hope is that the implementation of mTOR inhibitors into immunosuppressive regimes will reduce the risk of recurrence and development of secondary malignancies. Although recent evidence suggests a beneficial effect of mTOR inhibitors in the context of transplantation for malignant disease, a number of questions regarding this issue remain to be answered[16,17].

An important problem in transplant oncology is the timing of liver transplantation. The current allocation algorithm in Eurotransplant (ET) and the United Network for Organ Sharing (UNOS) system is based on the Model for End-Stage Liver Disease

(MELD) score. The MELD score, which is calculated by creatinine, bilirubin, and the international normalized ratio, favors the sickest patients, is a disadvantage for patients listed for malignant disease as their hepatic and renal functions are usually compensated. For certain indications such as HCC, the MELD exception (or exceptional MELD in Eurotransplant) score has been introduced to overcome this drawback. However, the exceptions are currently not applicable to all indications for liver transplantation in malignant tumors. The increased use of living-donor liver transplantation (LDLT) to overcome the problem of prolonged waiting time and organ shortage is an option, but not the solution, for the problem of timing in transplant oncology even though LDLT has become a standard in various regions worldwide. The present review will focus on the current status of transplant oncology and summarize some of the results obtained for the most frequent indications in liver transplantation for primary and secondary malignant tumors.

PRIMARY LIVER CANCER

HCC

HCC is by far the most common primary liver cancer and accounts for more than 80% of primary liver tumors[18]. In addition, it is a major global health, burden being the fourth most common cause of cancer-associated mortality worldwide[19]. In about 80% to 90% of patients, HCC develops in a cirrhotic liver. The main risk factors for cirrhosis and subsequent development of HCC are hepatitis B and/or C infection, alcohol, autoimmune liver disease, and hemochromatosis as well as nonalcoholic steatohepatitis (NASH), which is becoming more and more important[20,21]. In patients with preserved liver function, liver resection is the treatment of choice mainly because of the shortage of organs[22]. Nonetheless, several studies that compared liver resection and liver transplantation reported a superior outcome for liver transplantation regarding RFS after 3 and 5 years, and OS after 10 years[23,24]. Although some patients with HCC in cirrhosis are prioritized through MELD exception, a number of patients still drop out following tumor progression while on the waiting list [25,26]. Nonetheless, liver transplantation is regarded as the best treatment option for HCC in cirrhotic livers as it cures both the tumor and the underlying liver disease.

Patient selection is crucial in liver transplantation for HCC. In this regard, the Milan criteria, established by Mazzaferro *et al*[5] in 1996, represent the gold standard for HCC. The criteria include patients with a single HCC lesion up to 5 cm in diameter or with 2 to 3 HCC lesions up to 3 cm in diameter without macrovascular invasion or extrahepatic tumor growth. Five-year OS after transplantation for patients within the Milan criteria is reported to be around 70% with 10% to 15% recurrence rates[5,27]. Although initially defined in a series of only 48 patients, the Milan criteria are nowadays widely accepted and have been adopted by many guidelines including those of the European Association for the Study of the Liver-European Organization for Research and Treatment of Cancer (EASL-EROTC), the American Association for the Study of Liver Diseases (AASLD), as well as by Asian guidelines. With growing experience in liver transplantation for HCC, the strict Milan criteria were extended by several groups. Yao *et al*[28] introduced the University of California San Francisco (UCSF) criteria in 2007[28]. The criteria comprise a single lesion up to 6.5 cm in diameter or two to three lesions with up to 4.5 cm for the largest, and a maximum tumor diameter up to 8 cm[28]. Soon afterwards, even the Milan group extended their criteria to the so-called "up-to-seven criteria" (the sum of the number of tumors and diameter of the largest tumor of up to 7 cm)[29]. Additional criteria have been published by several groups from all over the world[30-35].

Over the years, significant efforts have been made to include parameters to address differences in tumor biology into the selection criteria. Markers such as tumor differentiation[36,37], des-γ-carboxy prothrombin (DCP)[38] and FDG-PET imaging[39] have been proposed. Notably, alpha-1-fetoprotein (AFP) is the most widely accepted biomarker for HCC, and an AFP level above 1000 ng/mL has been identified as a surrogate marker for vascular invasion and a significant predictor for tumor recurrence after transplantation[40]. More recently, AFP was included in new, expanded criteria for LDLT candidates with HCC in Japan. These so-called "5-5-500 criteria" (nodule size ≤ 5 cm, nodule number ≤ 5 and AFP ≤ 500 ng/mL) were established based on a retrospective analysis of the Japanese nationwide survey. When applying the criteria, a recurrence rate of only 7.3% was observed after 5 years, while the number of eligible patients was increased by 19% compared with the conventional Milan criteria [41]. Interestingly, Korean groups published encouraging results for liver transplan-

tation in cases with tumor-associated portal vein thrombosis, which is usually considered to be an absolute contraindication for liver transplantation[42,43]. In particular, Lee and coworkers reported a 5-year OS of around 63% and a 5-year RFS of around 45% in 11 patients who underwent liver transplantation[43]. These are undoubtedly impressive results in patients with advanced malignancies. However, as the criteria for organ allocation regarding liver transplantation in HCC are much more restrictive in many areas of the world, the results are hardly transferable particularly to Western countries. The most commonly used selection criteria for liver transplantation in HCC are summarized in Table 1.

In 2007 Mazzaferro[44] summarized a number of selection criteria, defined at that time in the so-called Metroticket. The principle of the Metroticket is that the greater the number or the larger the size of tumor nodules (in the words of the Metroticket, "the longer the trip"), the lower the expected patient survival (in the words of the Metroticket, "the higher the price")[44]. While the initial model was based on the conventional criteria, tumor number and size, a group from Italy and China defined the Metroticket 2.0 several years later by including AFP as a biological surrogate in the prediction model[45]. Results show that a 70% chance of HCC-specific 5-year OS can be obtained if the AFP level is below 200 ng/mL and the sum of the number and size (cm) of the tumors does not exceed seven. With an AFP level of 200-400 ng/mL prior to transplantation, the tumor number and size (cm) should not exceed five, and in cases with an AFP between 400 and 1000 ng/mL, the number and size (cm) should not exceed four. Very recently, the latter prediction model was again refined by adding the response to neoadjuvant therapies as determined by modified Response evaluation criteria in solid tumors (RECIST) criteria. Results were stratified by the last radiological staging before liver transplantation. To maintain the HCC-related deaths below 30% within 5 years from transplantation, Metroticket 2.0 criteria had to be modified for patients with a partial response (PR) or stable disease and also for progressive disease (PD)[46]. Taken together, the Metroticket model including the upgrades can help to estimate the risk of recurrence and cancer-related death more precisely.

To overcome the problem of binary decision systems, continuous-risk scores were subsequently developed. Particularly, Sasaki *et al*[47] described the Hazard Associated with Liver Transplantation for Hepatocellular Carcinoma (HALT-HCC) score, which includes the tumor burden score, AFP, and MELD-Na after initial evaluation of eight variables. This score was validated and recalibrated by an international study group using data from more than 4000 patients[48]. After recalibration, HALT-HCC increased in its prognostic utility regarding RFS and OS. Finally, Goldberg *et al*[49] recently published the Liver Transplant Expected Survival (LiTES)-HCC score, which emphasizes the issue that the majority of deaths after transplantation for HCC are not related to HCC recurrence. In addition, the etiology of liver disease is adjusted to the U.S. population (*e.g.*, the fraction of patients with NASH is higher). 11 variables were included and four groups based on the LiTES-HCC score were defined. Survival analysis showed a 1-year OS of 97% in the best group *vs* 90.2% in worst group, which were more pronounced with longer follow-up (5-year OS of 86.3% *vs* 67% and 10-year OS of 72.7% *vs* 47.7%)[49]. The data have the potential to change the current practice in prioritization of patients with HCC.

So far, the Milan criteria are the basis of selection for liver transplantation in numerous countries. However, there is a considerable risk of tumor progression for patients while on the waiting list, emphasizing the need for therapy as bridging until a suitable donor is available. The dropout rate without liver-directed treatment has been described as high as 25% after 6 mo and 38% after 1 year compared with 8.7% and 22.9% with locoregional therapy[25,26]. Usually, locoregional therapies including transarterial chemoembolization, local radiofrequency or microwave ablation, radioembolization, liver resection or stereotactic body radiation therapy are employed for bridging[25,50]. The choice of a certain locoregional therapy is based on liver function, size and number of tumors as well as institutional experience because of a lack of prospective randomized controlled trials comparing certain modalities before liver transplantation. An analysis of 18 studies by Kulik *et al*[50] found no significant impact on post-transplant survival or recurrence by pretransplant bridging therapy. Nonetheless, a recent subgroup analysis from the SiLVER study showed that patients who are progressing upon bridging have poorer OS compared with those with controlled disease[51].

Locoregional therapies can also be applied to decrease the number and/or size of tumors. This concept of downstaging to predefined criteria (usually the Milan criteria) is performed in many areas worldwide but not allowed in certain countries such as Germany. The success rate of downstaging to within the Milan criteria exceeds 40% as shown by Parikh *et al*[52] in a systematic review and pooled data analysis. Several

Table 1 Selection criteria for liver transplantation in hepatocellular carcinoma

Criteria	Definition	Overall survival, (%)	Comment
Milan criteria (MC) (1996)[5]	Single tumor ≤ 5 cm 2-3 tumors ≤ 3 cm No macrovascular involvement No extrahepatic disease	75% (4-yr OS) 92% (4-yr OS; inside MC at pathological examination)	Gold standard Validated by many subsequent studies
UCSF (2001)[28,163]	Single tumor ≤ 6.5 cm 3 tumors all ≤ 4.5 cm with total tumor diameter ≤ 8 cm	90% (1-yr OS) 75% (5-yr OS)	Initial definition on pathological examination Subsequently validated in preoperative imaging
Up-to seven (2009)[29]	Sum of the number of tumors and diameter of the largest tumor (in cm) ≤ 7	64% (beyond MC but within up-to-seven) Without microvascular invasion: 71% With microvascular invasion 47%	
Navarro criteria (2008)[32]	1 tumor ≤ 6 cm 2-3 tumors ≤ 5 cm	92% (1-yr OS) 73% (5-yr OS)	
Valencia criteria Silva *et al*[34] (2008)	Up to 3 tumors, each ≤ 5 cm, and a cumulative tumor burden ≤ 10 cm	69% (5-yr OS) Recurrence probability: 12% and 28% after 1 and 5 yr, respectively	
TTV (2008)[164]	TTV ≤ 115 cm^3 AFP ≤ 400 ng/mL[165]	80% (5-yr OS)	In some centers only LDLT for those beyond MC
ETC (2011)[31]	No limits in size and number No vascular invasion No extrahepatic disease No cancer-related symptoms Biopsy of the largest lesion not poorly differentiated	68% (beyond MC but inside ETC) 68% (5-yr OS)[37]	
Asan criteria (2008)[33]	Largest tumor diameter ≤ 5 cm Number of HCC lesions ≤ 6 No vascular invasion	76% (5-yr OS)	LDLT
Kyoto criteria (2007)[166]	Number of lesions ≤ 10 Size of the largest lesion ≤ 5 cm DCP ≤ 400 mAU/mL	Beyond MC but within Kyoto criteria: 65% 5-yr OS 82% (5-yr OS)[38]	LDLT
Kyushu criteria (2011)[167, 168]	Any number of tumors with a maximum diameter ≤ 5 cm DCP < 300 mAU/mL	- 81% (5-yr OS)[169]	LDLT
Tokyo criteria 8 (5-5 rule)[30, 35]	Up to 5 tumors Maximum diameter ≤ 5 cm	80% (5-yr OS)	LDLT
Samsung[170]	Maximum tumor size ≤ 6 cm Number of tumors ≤ 7 AFP ≤ 1000 ng/mL	90% RFS after 5 yr	LDLT
5-5-500[41]	Up to 5 tumors Maximum diameter ≤ 5 cm- AFP ≤ 500 ng/mL	- 76% (5-yr OS)	LDLT
Portal vein invasion			

Seoul National University Hospital[43]	PV tumor thrombus does not extend into the main PV AFP × DCP score ≤ 20000 Additional risk factors for recurrence: Tumor size > 7 cm Preop. FDG-PET SUV ratio ≥ 2.1 Vp4 infiltration	11 patients 64% (5-yr OS) 46% (5-yr RFS)	LDLT

AFP: Alpha-fetoprotein; DCP: Des-γ-carboxy prothrombin; ETC: Extended Toronto criteria; FDG: Fluorodeoxyglucose; HCC: Hepatocellular carcinoma; LDLT: Living-donor liver transplantation; MC: Milan criteria; OS: Overall survival; PV: Portal vein; PET: positron emission tomography; RFS: Relapse-free survival; SUV: Standardized uptake value; TTV: total tumor volume; UCSF: University of California San Francisco.

studies have reported that the survival of patients who were successfully downstaged is similar to those who were always within the Milan criteria[53]. Kardashian *et al*[54] reported evidence that successful downstaging was predicted by waiting time, AFP response to locoregional therapy, and tumor burden, which in turn provided excellent outcomes after liver transplantation. Furthermore, Mazzaferro *et al*[55] published results from a phase IIb/III trial showing that liver transplantation improves survival after effective and sustained downstaging to within the Milan criteria compared with nontransplant therapies. Finally, novel systemic treatment options based on the use of immune checkpoint inhibitors have recently been approved for treatment of advanced-stage HCC [56,57]. However, there is almost no literature available regarding their use in neoadjuvant setting before liver transplantation. A major concern when using those drugs in the transplant setting is the risk of organ rejection and death following hyperactivation of the immune system[57]. Experience so far is limited to case reports. Schwacha-Eipper *et al*[58] recently published a case of successful liver transplantation after neoadjuvant use of nivolumab while two other case reports indicate fatal hepatic necrosis following treatment with an immune checkpoint inhibitor, based therapy prior to transplantation[59,60]. Hence, the exact role and handling of these novel agents in neoadjuvant strategies before liver transplantation remains to be elucidated[57]. Nonetheless, the data on expanding the criteria to patients who were downstaged to within the Milan criteria seems to be convincing.

Regarding immunosuppression following liver transplantation for HCC, a recent systematic review and meta-analysis of 23 studies concluded that mTOR inhibitor-based immunosuppression is preferable, due to reduction of recurrence rate and improved RFS over at least 3 years[61]. That is further supported by earlier clinical data showing that reduction of CNI-based immunosuppression (> 10 ng/mL tacrolimus or > 300 ng/mL cyclosporin) reduces recurrence of HCC[62]. Evidence was also provided by a prospective randomized phase III trial (the SiLVER study) showing better RFS and OS at 3 to 5 years in HCC patients receiving immunosuppression with sirolimus compared with standard CNI-based immunosuppression, although the primary endpoint of the study (long-term RFS beyond 5 years) was not met[17]. A recent subgroup analysis from the SiLVER study emphasized that sirolimus treatment is most beneficial in patients with active HCC as determined by elevated AFP levels [63]. However, when using mTOR inhibitors for immunosuppression, the side effect profile has to be balanced with higher rates of proteinuria, peripheral edema, and incisional hernia on the one hand and preserved renal function on the other hand[64]. Nonetheless, some evidence supports the use of mTOR inhibitors as immunosuppression after liver transplantation for HCC.

Taken together, selection is crucial in patients with HCC who are scheduled for liver transplantation. Although the Milan criteria are still the standard in most countries, several efforts have been made to refine and extend the inclusion criteria for liver transplantation in HCC. Bridging therapies while being on the waiting list are commonly used, and downstaging strategies are performed in most countries. Finally, the optimal immunosuppression after liver transplantation for HCC is still a matter of ongoing research.

Fibrolamellar carcinoma

Fibrolamellar carcinoma (FLC) is a rare liver tumor that accounts for less than 1% of primary liver tumors[65]. Initially regarded as a subtype of HCC, this tumor entity has received its own classification code from the World Health Organization in 2010[66].

FLCs are usually detected in younger patients between 10 and 30 years of age but a second peak has been reported in patients between 60 and 69 years of age[65]. As the tumor arises without an underlying liver disease, liver resection including lymphadenectomy is the preferred treatment, with 5-year OS between 50% and 70%[67-69]. Liver transplantation is considered in selected cases with unresectable FLC. However, patients with FLC do not get a MELD exception, which often precludes them from liver transplantation because of the usually preserved liver function. Because of the low incidence and the possibility to perform extended liver resection, experience with liver transplantation is limited to case reports or small case series. Atienza *et al*[70] analyzed data from 63 patients from the UNOS database who underwent liver transplantation between 1988 and 2013. 1-, 3- and 5-year OS were 90%, 80% and 48%, respectively, with a 10% recurrence rate. Mavros *et al*[68] summarized data from 484 patients, of whom 109 underwent liver transplantation. Survival was reported in six studies with 1-, 3-, and 5-year OS between 63%-100%, 43%-75%, and 29%-55%, respectively. Unlike HCC with coexistent cirrhosis, no selection criteria for liver transplantation have been defined for FLC. Therefore, results from the case series have to be handled with caution. Nonetheless, given the acceptable outcome published in the aforementioned reports, liver transplantation seems to be a treatment option in selected patients with FLC who are not candidates for liver resection.

Combined hepatocholangiocarcinoma

Combined hepatocholangiocarcinoma (HCC-CC) is also rare, accounting for 0.75% to 1% of primary liver tumors. The incidence is reported to be around 0.5 per 1,000,000 and has been slightly increasing in the last decades[71]. Because of difficulties in preoperative imaging, the diagnosis of combined HCC-CC is usually made in the postoperative pathological report, and even if suspected intraoperatively, confirmation of combined HCC-CC is often difficult in frozen sections. Liver cirrhosis is detected in more than 50% of cases. So far, liver resection is the treatment of choice, if liver function is preserved[72]. Experience with liver transplantation is limited to small case series or case reports. In 2013, Groeschl *et al*[73] compared patients who underwent either resection or transplantation for HCC and HCC-CC. Results showed that liver transplantation for HCC-CC had a 3-year OS (48%) similar to liver resection (46%) but was inferior to transplantation for HCC (78%)[73]. Garancini *et al*[74] analyzed data from 485 patients in the Surveillance, Epidemiology, and End Results (SEER) database who were treated for HCC-CC between 1988 and 2009. Among them, 13% underwent liver transplantation with 5-year OS of 41%[74]. A recent report from 19 patients from Germany also found a 1-year OS of 57% and a 5-year OS of 38%[75]. In contrast to these disappointing results, Sapisochin *et al*[76] analyzed data from 15 patients with HCC-CC and 30 patients with HCC in a retrospective 1:2 matched cohort analysis. Results showed no difference when comparing the OS rates of liver transplantation for HCC-CC (1-, 3-, 5-year OS: 93%, 78%, 78%, respectively) with the matched group of HCC (1-, 3-, 5-year OS: 97%, 86%, 86%, respectively. Similarly, a series from Korea described encouraging 1- and 5-year OS of 84% and 66%, respectively, in 32 patients who were transplanted between 2005 and 2014. Notably, the recurrence rate was 16% after 1 year and 32% after 5 years. The authors also found that patients with very early stage combined HCC-CC (1 or 2 tumors ≤ 2.0 cm) had only 13% tumor recurrence and as much as 93% OS at 5 years[77]. De Martin *et al*[78] described a group of 49 patients with either intrahepatic cholangiocarcinoma (iCC) (n = 24) or HCC-CC (n = 25) who received liver transplantation. Overall, 1- and 5-year survival were 90% and 67%, respectively. When comparing iCC and HCC-CC, OS and RFS in the two groups were comparable[78]. In line with those results, Dageforde *et al*[79] analyzed data from more than 3000 patients with HCC (n = 2998) or HCC-CC (n = 208) who underwent either liver resection or liver transplantation in an American multicenter analysis. In the subgroup of patients within the Milan criteria, 67 with HCC-CC underwent liver transplantation. Those patients had similar OS compared to patients who underwent transplantation for HCC (5-year OS 70% for HCC-CC *vs* 73% for HCC) despite higher recurrence rates (23% with HCC-CC *vs* 12% with HCC at 5 years)[79]. The largest registry data comprises 220 patients from the National Cancer Database who underwent liver transplantation for HCC-CC and reported a 5-year OS of 52%[80]. However, the aforementioned results are still not conclusive. Therefore, a recent working group manuscript from the International Liver Transplantation Society (ILTS) Transplant Oncology Consensus Conference states that there is still no consensus about the role of liver transplant in the treatment of HCC-CC[81].

iCC

iCC is the second most common primary liver tumor and its incidence is increasing worldwide, especially in Western countries[82-84]. This tumor can arise in both, noncirrhotic and cirrhotic livers. To date, liver resection with lymphadenectomy is the treatment of choice, in cases of resectable disease. iCC is considered to be a contraindication for liver transplantation because of poor OS and RFS results reported in historical data[85,86]. In fact, several case series report 5-year OS rates between 0% and 30%[86-90]. However, in the last decade some encouraging results were published. A very recent multicenter study from Japan including 19 patients with incidentally detected iCC reported tumor recurrence in 10 patients (53%). OS at 1-, 3- and 5-years was 79%, 63% and 46% while RFS was reported to be 79%, 45% and 45%, respectively [91]. Furthermore, Sapisochin *et al*[76] performed a retrospective matched cohort study of incidentally detected iCC showing 51% 5-year OS and 5-year RFS of 36% in 27 patients. The results were inferior compared with the control group of HCC, but was still better than in previous reports. Moreover, the authors confirmed their results in a larger multinational study with 48 patients. In particular, patients with single iCC of up to 2 cm in diameter who underwent liver transplantation (n = 15) had a 5-year OS of 65% and an RFS of 82%. In contrast, patients with tumors larger than 2 cm or multiple lesions (n = 33) had a 5-year OS of 45% and RFS of 39%[92]. Similar results were reported by Facciuto *et al*[93], with a 5-year OS of 57% and an RFS of 57% in seven patients. In that study, patients with iCC features within the Milan criteria had a tumor recurrence of only 10% and a 5-year survival of 78%, which is comparable to patients with HCC within Milan criteria. However, the studies mentioned so far included almost exclusively patients with incidentally detected iCC. Currently, a prospective phase II trial from Canada is evaluating the impact of liver transplantation in patients with very early iCC (≤ 2 cm) and 5-year OS as the primary endpoint (NCT02878473).

In contrast, Lunsford *et al*[94] prospectively analyzed the impact of neoadjuvant gemcitabine-based chemotherapy in the context of liver transplantation for iCC. Patients had to be stable for 6 mo before getting on the waiting list for transplantation. Of 12 patients enrolled in the protocol, six finally underwent liver transplantation. OS at 1-, 3-, and 5-years was 100%, 83%, and 83%, respectively. Three patients (50%) developed recurrence after a median of 7.6 mo while the other three patients remained free of recurrence (5-year RFS 50%)[94]. Finally, Wong *et al*[95] recently reported results from a prospective pilot study of neoadjuvant therapy for downstaging of locally advanced cholangiocarcinoma prior to liver transplantation. Of 18 patients who started neoadjuvant treatment, 11 dropped out because of tumor progression or uncontrolled infection. Five received transplantation, with two of them suffering from iCC. The 1-year OS was 80%, three patients were recurrence free, and one developed tumor recurrence[95]. A very recent systematic review and meta-analysis by Ziogas *et al*[96] summarized the available data from 355 patients in 18 studies. Pooled 1-, 3- and 5-year OS was reported to be 75%, 56%, and 42%, while pooled 1-, 3-, 5-year RFS was 70,%, 49%, and 38%, respectively. However, in the subgroup with very early iCC (a single tumor, ≤ 2 cm) 5-year RFS was 67% compared with 34% in more advanced stages[96]. Taken together, although encouraging results have been reported for patients with a single iCC ≤ 2 cm and those who are stable or respond to a neoadjuvant therapy, liver transplantation for iCC is still not a standard of care and should best be performed in the context of clinical trials.

Perihilar cholangiocarcinoma

Perihilar cholangiocarcinoma (pCC) accounts for about 50% of all cholangiocarcinomas. The tumor develops either without liver disease or on the basis of primary sclerosing cholangitis. Radical resection is the state-of-the art therapy and provides a 5-year OS of between 20% and 50% in cases with R0 resection[97,98]. However, a significant proportion of patients cannot undergo resection either because of distant metastases, a locally advanced tumor, quality of liver parenchyma, or underlying liver disease[99]. Hence, liver transplantation might be the only remaining curative option for those patients. Initial results published 20 to 30 years ago were poor, with high recurrence rates and 5-year OS between 0% and 25%[86,100]. Almost two decades ago, more encouraging results were published by De Vreede *et al*[101] from the Mayo Clinic and Sudan *et al*[102] from Nebraska. Both studies employed neoadjuvant treatment including brachytherapy and systemic chemotherapy with or without external beam radiation. In addition, strict criteria for patient selection were introduced (*e.g.*, no extrahepatic disease, no lymph node metastases as determined by lymphadenectomy.). Results were impressive, with tumor recurrence in one of 11

patients in the study by De Vreede *et al*[101] and two of 11 patients in the study by Sudan *et al*[102]. Subsequently, a number of case series were published confirming this data. Cambridge *et al*[103] very recently summarized the evidence in a meta-analysis and meta-regression of survival after liver transplantation for unresectable pCC. Results from 20 studies including 428 patients showed pooled 1-, 3-, and 5-year OS of 77%, 55%, and 45%, respectively. However, when neoadjuvant chemoradiotherapy was employed, OS improved to 83%, 66%, and 65% at 1, 3, and 5 years, respectively. In addition, the recurrence rate was as low as 24% after 3 years with neoadjuvant treatment compared to 52% when no neoadjuvant treatment was applied[103]. An important issue is the percentage of patients who undergo neoadjuvant chemoradiotherapy but do not proceed to transplantation. Several studies report that the dropout rate ranges between 0% and 66%, mostly for local or distant tumor progression[104-108]. The impressive results obtained by the neoadjuvant therapy and subsequent liver transplantation led to the introduction of a MELD exception for pCC in the U.S. (UNOS) and also in Eurotransplant[109,110]. Nonetheless, prioritization is currently performed similar to HCC which in turn leads to higher waitlist drop out in patients with pCC[111]. Hence, refinement of the current practice is warranted.

Another important question is whether resection or transplantation should be the preferred treatment of pCC. Regarding that issue, the group from Nagoya published a 5-year OS of 53% after the resection of pCC without nodal metastases, emphasizing a similar outcome compared to liver transplantation after neoadjuvant chemoradiation [98]. In contrast, Ethun *et al*[112] analyzed data from 191 patients who underwent resection and 41 who underwent transplantation for pCC from 10 institutions. The results showed a benefit for transplantation regarding 3- and 5-year OS (72% *vs* 33% and 64% *vs* 18%, respectively). Even patients who underwent resection within the strict selection criteria for transplantation (a tumor < 3 cm, no lymph node metastases, no extrahepatic disease) had inferior outcomes compared with transplantation, with a 5-year OS of 54% *vs* 29%[112]. An ongoing multicentric randomized trial in France is comparing capecitabine-based chemoradiotherapy with subsequent liver transplantation to standard liver and extrahepatic bile duct resection (NCT022322932; TRANSPHIL). Regarding selection criteria, most case series included only patients with tumors ≤ 3 cm, absence of lymph node metastases, and without signs of distant metastases. The neoadjuvant concept was also recommended by a recent working group report from the ILTS Transplant Oncology Consensus Conference. In addition, a dominant stricture and at least either positive cytology from brush endoscopy or biopsy demonstrating pCC or elevated CA19-9 above 100 U/mL in the absence of cholangitis were suggested. Finally, the use of arterial and venous jump grafts in the setting of liver transplantation was advised[81].

Hepatic epithelioid hemangioendothelioma

Hepatic epithelioid hemangioendothelioma (HEHE) is a very rare primary liver sarcoma accounting for approximately 1% of all vascular tumors[113]. The incidence is reported to be around 1-2/1000000 with a predominance in females[114]. The natural course of HEHE is unpredictable, ranging from aggressive growth to indolent behavior[115,116]. The tumor affects only the liver in about 21% of patients. Most common tumor sites other than the liver are the lungs, bone, and lymph nodes[115]. Regarding treatment modalities, no consented algorithm exists for EHE. However, Lerut and Iesari suggested a treatment algorithm for HEHE in 2017, with liver transplantation playing a central role[113]. Regarding surgical therapy, Konstantinidis analyzed a cohort of liver sarcomas from the National Cancer Database who underwent surgery between 2004 and 2014. The subgroup of HEHE who underwent either liver resection or transplantation showed no significant difference in OS. However, results from several case series and analyses from national and international transplant databases suggest that liver transplantation provides a high survival advantage. In particular, Rodriguez *et al*[117] analyzed data from 110 patients in the UNOS database and found 1- and 5-year OS of 80% and 64%, respectively. Of note, about a quarter of the patients were younger than 4 years of age at the time of transplantation[117]. Lerut *et al*[118] used data from 59 patients in the European Liver Transplant Registry (ELTR) with 1-, 5-, and 10-year OS of 93%, 83%, and 72%, respectively. Interestingly, the authors found that extrahepatic disease (EHD) did not influence the outcome after transplantation (up to 78% OS after 10 years in cases of EHD)[118]. A follow-up analysis of 149 patients who underwent liver transplantation for HEHE between 1984 and 2014 confirmed the aforementioned results regarding 1-, 5- and 10-year OS (89%, 80%, and 74%, respectively). However, in that study, pretransplant waiting time less than 120 d, macrovascular invasion, and hilar lymph node invasion but no other extrahepatic manifestations were found to be associated

with significant risk of recurrence. Based on the three parameters of pathological macrovascular invasion, hilar lymph node positivity, and a waiting time of < 120 d, the authors calculated a score to estimate the risk of recurrence, with 93.9% 5-year disease-free survival (DFS) in the low-risk situation (≤ 2 points) compared with 38.5% 5-year DFS in the high-risk situation (≥ 6 points)[119]. A recent study by Brahmbhatt *et al*[120] showed comparable results after liver transplantation for HEHE and HCC (inside the Milan criteria). However, the authors observed an increased rate of graft failure because of arterial thrombosis in patients with HEHE within the first 14 d after transplantation[120]. In summary, liver transplantation can provide excellent long-term results in patients suffering from HEHE, even in the presence of EHD.

Hepatic angiosarcoma

Hepatic angiosarcoma (HAS) is a very aggressive mesenchymal liver tumor. Fortunately, with an incidence of 0.5-1 cases per 1000000 people, it is extremely rare. HAS gained some attention because of its association with environmental factors such as vinyl chloride, thorotrast, radium, and cyclophosphamide[121]. According to a recent pooled analysis of the literature over the last 20 years, the prognosis of HAS is very poor. The review included 219 patients with a median survival of 6 mo and 1- and 2-year OS of 30%, and 17%, respectively[122]. If possible, liver resection is the treatment of choice. Some small series report a median OS between 17 and 19 mo, which can be extended in case of R0 resection[122,123]. Regarding liver transplantation only small case series have been reported. A retrospective analysis of 22 patients from the ELTR in 2013 found that no patient survived longer than 23 mo after transplantation. Indeed, recurrence was diagnosed after a median of 5 mo and almost 80% of patients died of recurrence[124]. Interestingly, in about 70% of patients, the diagnosis of HAS was not known prior to transplantation. Similar results, a median OS of 6 mo, were reported in a study by Konstantinidis *et al*[125]. However, some case reports have been published showing at least some success with liver transplantation and subsequent adjuvant chemotherapy and/or mTOR inhibitor-based immunosuppression[126-129]. Nonetheless the data do not affect the overall dismal results after liver transplantation for HAS. As a consequence, HAS is regarded as a contraindication for liver transplantation in Europe and the US[124,130].

Undifferentiated embryonal sarcoma of the liver

Undifferentiated embryonal sarcoma (UES) is a very rare indication for liver transplantation. The tumor was first described by Stocker and Ishak in 1978[131]. In fact, UES of the liver (UESL) in mainly diagnosed in children between 6 and 10 years of age and it accounts for 1%-4% of all solid childhood tumors[132,133]. Surgical resection with or without chemotherapy is currently recommended for therapy of this tumor[133,134]. Very recently, Babu *et al*[135] summarized the experience with liver transplantation for UESL. Only 28 cases we reported, among them only four patients were 18 years of age or older[135]. Notably, the oldest patient was described by Dhanasekaran *et al*[136] in 2012. The patient underwent liver transplantation in 2002. Although retransplantation was necessary following ductopenic rejection, he was tumor free for more than 10 years after the second transplantation[136]. In summary, although very rare, the option of liver transplantation for UESL should be kept in mind even in adults when liver resection is not possible.

SECONDARY LIVER CANCER

Liver transplantation for secondary malignant liver tumors was initially investigated more than 30 years ago. As results from early case series were poor, with 5-year OS between 0% and 20%[137-139], liver transplantation for this indication has been abandoned for years, with liver metastases from neuroendocrine tumors being the only exception. However, for liver metastases of colorectal cancer, the refusal of liver transplantation as a therapeutic option has diminished over the last decade.

NECLM

Over the years, NECLM was the only accepted indication for liver transplantation in patients with secondary liver tumors. However, the clinical course of patients suffering from neuroendocrine tumors (NET) is extremely variable and mainly dependent on differentiation. While low-grade NET (G1/G2) often has a slow-growing, indolent clinical course, High-grade tumors are usually more aggressive. Most NETs are located in the gastroenteropancreatic system, with pancreas being the

most frequent location within that area[140]. Regarding the primary tumor location, liver transplantation for NETs other than those from gastroenteropancreatic system are considered to be a relative contraindication by some groups, although 5-year OS was 53% in 16 patients who underwent liver transplantation after resection of a pulmonary NET in an ELTR study[141]. Evaluation of distant metastases outside the liver is important in NET with octreotide or gallium-68 DOTATATE or DOTATOC-PET being the most sensitive examinations[140]. The liver is the only metastatic location in about 50% of patients but only 0.2%-0.3% of all liver transplantations are performed for NECLM. Variability in the clinical course and the presence of a wide range of treatment options (*e.g.*, liver resection, local ablation, peptide receptor radionuclide therapy, transarterial approaches, and medical therapies) makes it difficult to define the optimal place and timing for liver transplantation in the therapy algorithm of NECLM[140]. However, the largest systematic review that summarized data on liver transplantation for NECLM between 1974 and 2016, comprised more than 1,000 patients[142]. 1-, 3-, and 5-year OS was 89%, 69%, and 63%, respectively. The recurrence rates after transplantation ranged from 31% to 57%. The largest study in the review was conducted by Le Treut *et al*[141] and was based on 213 patients included in the ELTR over a 27-year period. The authors found a 5-year OS and DFS from the time of liver transplantation of 52% and 30%, respectively[141]. Three factors associated with adverse outcomes were identified, simultaneous major resection (*e.g.*, primary tumor resection), poor differentiation (G3/4) and hepatomegaly. In 2007 Mazzaferro *et al*[143] had already defined selection criteria for liver transplantation of NECLM, the so-called "Milan NET" (Table 2). By applying these criteria, the group from Milan published results from 42 patients who underwent liver transplantation with impressive 5- and 10-year OS of 97% and 89%, respectively. Survival rates were significantly higher than those of 46 patients with a similar tumor burden who did not undergo liver transplantation (5-, 10-year OS 50.9% and 22.4%, respectively). Of note, the recurrence rate following liver transplantation in this study was only 13%, which is comparable to that of HCC patients undergoing liver transplantation within the established criteria[144]. However, some of the selection criteria used in the Milan NET score are debatable (*e.g.*, the cutoff age of 55 years). The UNOS guidelines and a revision of the Milan NET from 2016 advise an age cutoff of 60 years[144,145]. Another important issue is the use of certain immunosuppressants after liver transplantation for NECLM. mTOR inhibitors have been used for both immunosuppression after liver transplantation and for the treatment of NET[17,140,146]. Hence, the obvious hope is that using mTOR inhibitors as an immunosuppressant might reduce recurrence rates. In summary, although numerous questions remain to be answered, liver transplantation has the potential to improve the prognosis of patients suffering from NECLM.

CRLM

Colorectal cancer is one of the most common tumor entities worldwide[147]. About 50% of patients develop liver metastasis, a major predictor of OS. The current mainstay for the treatment of CRLM is surgical resection if possible as a 5-year OS of around 30% to 60% can be reached even in advanced stages. However, only 20% to 30% of patients with CRLM are eligible for surgery[148,149]. In cases of irresectable disease, the prognosis dramatically decreases even with novel chemotherapeutic regimes[150-152]. The group from Vienna was among the first to report long-term results of liver transplantation for CRLM. Twenty-five patients underwent transplantation, and the reported 5-year OS was only 12%[137]. In 2013, Hagness *et al*[153] published first results of the SECA-I trial including 21 patients with irresectable liver metastases from colorectal cancer who underwent transplantation. The authors reported an impressive OS of 95%, 68%, and 60% at 1, 3, and 5 years, respectively. DFS at 1 year was as low as 35%, but only six of 21 patients died after a median 26 mo after transplantation because of disseminated cancer progression[153]. The results show that recurrence after transplantation was quite common but treatment of post-transplant recurrence using chemotherapy or surgery was comparatively effective. Nonetheless, the report by the Oslo group brought new enthusiasm to the issue of liver transplantation in CRLM. In particular, the OS reported by Hagness *et al*[151,154] was far superior to that obtained by chemotherapy alone. More recently, the same group published even better results from the follow-up SECA-II study, including 15 patients with irresectable CRLM. The 1-, 3- and, 5-year OS were 100%, 83%, and 83%, respectively. Furthermore, the DFS was improved compared with the SECA-I trial with 53%, 44%, and 35% at 1, 2 and 3 years, respectively[155]. Since then, several other groups have reported their experiences with liver transplantation for CRLM over the past years. Giannis *et al*[156] performed a pooled data analysis comprising 18 studies with 110 patients showing a 5-

Table 2 2007 Milan neuroendocrine tumor criteria[143]

Inclusion criteria
Histology G1/G2 neuroendocrine tumor with or without syndrome
Primary tumor drained by the portal system (pancreas and intermediate gut: from distal stomach to sigmoid colon)
Primary tumor removed with a curative resection (pretransplant removal of all extrahepatic tumor deposits) through surgical procedures separate from transplantation
Less than 50% tumor load in the liver parenchyma
Response to treatment or stable disease for at least 6 mo pretransplantation
Age younger than 55 yr (relative criteria)
Exclusion criteria
High-grade neuroendocrine carcinomas
General contraindications for liver transplantation, including previous tumors
Non-gastrointestinal NET or tumors not drained by the portal system

NET: Neuroendocrine tumor.

year OS of 51%. Of note, patients who underwent liver transplantation after 2005 had a 5-year OS of 66%, but the reported RFS after 5 years was similar compared with the period before 2005 (26%)[156].

A crucial point in liver transplantation for CRLM is careful patient selection. Kappel *et al*[157] found the detection of micrometastases in lymph nodes of the primary tumor to be associated with impaired survival after liver transplantation for CRLM. The latter SECA-I study had rather broad and arbitrary inclusion criteria: irresectable liver metastases, complete resection of the primary tumor, good performance status (ECOG 0 or 1), completion of 6 wk of chemotherapy. Patients were excluded in case of a weight loss of > 10%, extrahepatic tumor growth, or general contraindications for liver transplantation. In subsequent analyses, a Norwegian group identified risk factors for inferior outcomes. The factors included a tumor diameter > 5.5 cm, CEA level > 80 μg/L, time lapse of < 2 years from resection of the primary tumor to liver transplantation, and progression while on chemotherapy. Hagness *et al*[153] summarized those four parameters in the so-called Oslo score (1 point for each). For the SECA-II study, the inclusion criteria were more strict. Response to chemotherapy and at least 1 year from diagnosis of colorectal cancer to listing for liver transplantation were required[155]. Moreover, the Oslo group reported that patients with an Oslo score of 2 or less had a 5-year OS of 67% compared with only 17% in patients with an Oslo score of 3 or 4[158]. In addition, the primary tumor seems to be of particular importance for patient selection. Right-sided tumor location, *BRAF* mutation, and signet ring cell carcinoma are associated with poor outcome similar to the data from liver resection[158-161]. Furthermore, lymph node status of the primary seems to have some relevance although that is not an independent prognostic factor [159,160]. Remarkably, the group from Oslo recently published data comparing the results after PVE and subsequent liver resection with those of liver transplantation for CRLM[159]. Analysis of the subgroup of patients with a high tumor load determined as > 9 metastases and a high tumor burden score, showed a survival advantage for patients who underwent liver transplantation (median survival of 40.5 mo with liver transplantation *vs* 19.2 mo with PVE and resection). Of course, these impressive results have to be confirmed, but nonetheless harbor the potential to change the current management for CRLM. Regarding patient selection, the ILTS Transplant Oncology Consensus Conference working group gave recommendations for the use of liver transplantation in patients with CRLM. They include only liver involvement, an Oslo score ≤ 2, minimization of immunosuppression, and aggressive treatment of recurrence after transplantation[162].

The issue of immunosuppression after liver transplantation for CRLM is a matter of ongoing discussion. The Oslo group used CNI-free mTOR inhibitor-based immunosuppression in the SECA-I and -II trials because of its anticipated antineoplastic effects. Rejection was observed in 38% of patients in the SECA-I study, which is a marked increase compared with 28% with calcineurin-inhibitor based immunosuppression in a historical cohort from the Oslo group[153]. However, patient numbers in

the above mentioned studies were low, and further studies are needed to determine whether treatment with mTOR inhibitors has an inhibitory effect on tumor recurrence after liver transplantation for CRLM.

Currently, a number of questions are being addressed by ongoing studies. The SECA-II study aims to report 10-year OS. Although initial results regarding 5-year OS have already been published, final results are expected in 2025. The Oslo group also launched the SECA-III study to compare liver transplantation with any other treatment including chemotherapy, ablation, SIRT or other available treatment options (NCT03494946). The study plans to recruit 30 patients and is scheduled to report in 2027 with 2-year OS from the time of randomization being the primary outcome parameter. Another ongoing study is TRANSMET, launched by a French group. The study compares liver transplantation followed by standard chemotherapy to standard chemotherapy alone and plans to recruit 90 patients. The primary endpoint is 5-year OS (NCT02597348). The study is recruiting and is scheduled to report in 2027. The issue of LDLT is currently being investigated by a group in Toronto. The study plans to include 20 patients with liver-only metastases to receive LDLT. The primary endpoints are 5-year OS and DFS. Of note, a main exclusion criteria in this study is the presence of BRAF-positive tumors (NCT02864485). A stepwise approach with partial hepatectomy and transplantation of segment II/III from deceased donors during the first operation, followed by (second step) hepatectomy in 4 wk, is being investigated again by the Norwegian group. This so-called RAPID trail will include 20 patients, and the primary endpoint is 5-year OS (NCT02215889). Finally, the LIVERT(W)OHEAL trial from two German centers is currently ongoing. The study combines LDLT of segment II/III with the aforementioned stepwise approach of partial hepatectomy + segment II/III transplantation during the first operation and completion hepatectomy during the second operation. Primary endpoints are 3-year OS and DFS after the second-stage hepatectomy in 40 patients (NCT03488953). Study end is supposed to be 2023.

These studies will certainly answer a number of questions, but even more remain to be addressed. For instance, the issue of adjuvant chemotherapy after successful liver transplantation, impact of certain mutations (*e.g.*, *SMAD4*) that are known to be associated with impaired survival, or the optimal immunosuppression regime, to name just a few. Furthermore, the dramatic organ shortage remains a major issue that can only partly be relieved by LDLT. Nonetheless, liver transplantation for CRLM will gain more and more attention in light of the above mentioned evidence. Based on published data and the results from ongoing studies, it is assumed to find its place in treatment armamentarium for CRLM.

CONCLUSION

Liver transplantation for malignant disease has become a part of the armamentarium of cancer therapy. In HCC and HEHE, liver transplantation is an established treatment option. Increasing evidence supports the use of liver transplantation in pCC after neoadjuvant therapy, whereas the outcome of transplantation for iCC is not yet well defined. In FLC, liver transplantation might be an option, while patients with HAS are not considered to be optimal candidates. Regarding secondary liver cancer, NECLM remain an indication in selected patients, and recent evidence strongly supports liver transplantation for liver-only CRLM, although optimal selection criteria have to be defined.

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MINIREVIEWS

TRIM25: A central factor in breast cancer

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Abstract

TRIM25 is emerging as a central factor in breast cancer due to its regulation and function. In particular, it has been shown that: (1) Estrogens modulate *TRIM25* gene expression; (2) TRIM25 has activity as an E3-ligase enzyme for ubiquitin; and (3) TRIM25 is also an E3 ligase for interferon-stimulated gene 15 protein in the ISGylation system. Consequently, the proteome of mammary tissue is affected by TRIM25-associated pathways, involved in tumor development and metastasis. Here, we discuss the findings on the mechanisms involved in regulating *TRIM25* expression and its functional relevance in breast cancer progression. These studies suggest that TRIM25 may be a biomarker and a therapeutic target for breast cancer.

Key Words: TRIM25; Interferon-stimulated gene 15; Ubiquitin; Estrogens; Breast cancer

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Core Tip: TRIM25 is an E3-ligase enzyme for the ubiquitination and ISGylation system. *TRIM25* expression is deregulated in breast cancer. The activity of TRIM25 is associated with changes in the proteome of mammary tumors. The molecular mechanisms of modulation and action of TRIM25 are implicated in breast cancer progression.

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INTRODUCTION

Approximately 85% of breast cancer cases are associated with a multifactorial beginning. Breast cancer has a high incidence and mortality among women. The majority of mammary tumors (> 80%) are invasive and grow mainly in the mammary ducts (invasive ductal carcinoma), and a minor fraction of them can also grow in the mammary lobules. The establishment of new biomarkers has been difficult because the molecular characteristics of mammary tumors are highly heterogeneous, defining breast cancer as a collection of mammary neoplasms[1-3].

More than 70% of all breast cancer cases are estrogen receptor alpha positive (ERα+), whereas approximately 30% are ERα-[4]. In addition to ERα, other biomarkers for breast cancer include the progesterone receptor (PR), androgen receptor, human epidermal growth factor receptor 2 (HER2), and Ki67 (a cellular marker for proliferation)[5,6]. Consequently, the classification of breast cancers is based on the detection of these biomarkers, resulting in four subtypes: (1) Luminal A-like (the most common): ERα+, PR ≥ 20%, HER2-, Ki67 < 20%; (2) Luminal B-like: ERα+, PR < 20% and/or HER2+ and/or Ki67 ≥ 20%; (3) HER2-overexpression: ERα-, PR-, HER2+; and (4) Basal-like: ERα-, PR-, HER2- (triple-negative). Luminal A is more responsive to endocrine therapy than luminal B. HER2-overexpressing subtypes are responsive to anti-HER2-targeted therapy; however, basal-like breast cancer is not responsive to these therapies[7,8].

Endocrine therapy includes selective estrogen receptor downregulators (SERD), selective estrogen receptor modulators (SERM), and aromatase inhibitors (AI)[9,10]. SERD and SERM negatively regulate the abundance and activity of ERα, respectively, whereas AI blocks estradiol production. These therapies are important because ERα is activated mainly by 17 β-estradiol to act as a transcription factor or as a mediator of signaling pathways. In breast cancer, the 17 beta-estradiol/ERα pathways have been associated with a pro-tumor activity. Despite the existence of endocrine therapies, a common problem in breast cancer management is de novo resistance or acquired resistance to endocrine therapy through yet unidentified mechanisms[11]. The panorama of breast cancer leads to the search for new biomarkers and molecular targets for therapies to control its progression. We reviewed TRIM25 as a potential biomarker and therapeutic target for breast cancer. TRIM25 is a protein affecting the proteome through its E3 ligase activity for interferon-stimulated gene 15 (ISG15) and ubiquitin, as described below.

ISGYLATION AND UBIQUITINATION

Ubiquitination and ISGylation are two processes that introduce posttranslational modifications to proteins by covalently adding ubiquitin (Ub) and ISG15 to target proteins, respectively (Figure 1). ISG15 is structurally related to the Ub, but its actions and mechanisms of regulation display differences[12,13].

Ub is an 8 kDa protein [76 amino acid (aa)] conserved from yeast to humans, which contains the LRLRGG motif in its C-terminal domain that mediates covalent binding with its target proteins during ubiquitination, which is executed by three enzymes: (1) E1-Ub activating enzymes; (2) E2-Ub conjugating enzymes; and (3) E3-Ub ligase enzymes. The E3-Ub ligases are responsible for providing substrate specificity, with more than 600 members in the ubiquitination system, one of them being TRIM25. Ubiquitination can be reverted by de-Ubiquitinases, which are approximately 100 different in humans. Some proteins can be monoubiquitinated in one or several lysine residues[13]. In addition, some proteins can be polyubiquitinated since the Ub lysine residues 6, 11, 27, 29, 33, 48, and 63 can be used for the sequential attachment of these proteins, forming homotypic Ub chains or branched Ub chains (by binding of several lysine residues of Ub) on their target proteins[14,15]. In general, monoubiquitination regulates DNA repair, gene expression, endocytosis, and protein stability, and polyubiquitination is a marker for degradation by the proteasome [ubiquitin-proteasome system (UPS)] and some non-proteolytic activities[16].

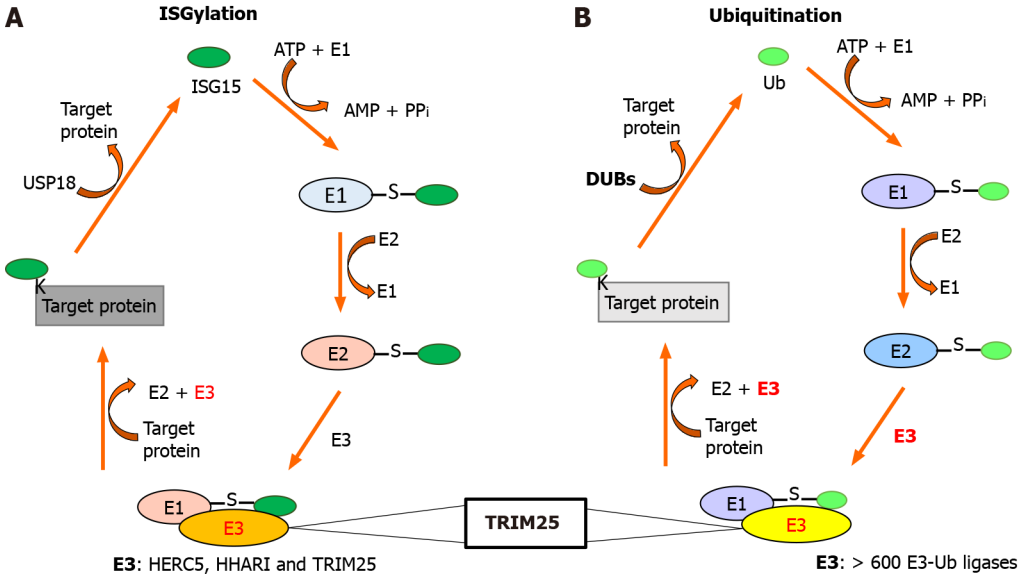


Figure 1 TRIM25 is an E3-ligase common for interferon-stimulated gene 15 and ubiquitin proteins. A: The ISGylation system; B: The Ubiquitination system. The ISGylation system and the ubiquitination system are similar, however, the ubiquitination system has more than 600 E3 ligases and the ISGyaltion system has three E3 ligases. Interestingly, both systems have in common the E3 ligase tripartite motif containing 25. ISG15: Interferon-stimulated gene 15; DUBs: Ubiquitin-proteasome system; Ub: Ubiquitin; USP18: Ubiquitin-specific protease 18.

In contrast to Ub, ISG15 is produced from a precursor protein of 165 aa, which is processed by removing eight aa at the C–terminal as well as the N-terminal methionine. Mature ISG15 (15kDa) has two Ub-like domains connected by a "hinge" sequence, and contains the motive LRLRGG in its C-terminal domain as Ub[17]. Similar to ubiquitination, ISG15 requires the E1 activating enzyme, the E2 conjugating enzyme, and the E3 ligase enzyme to covalently bind to lysine residues on its target proteins, denominating this process as ISGylation[18]. In contrast to the ubiquitination system, only three E3 ligase enzymes are known to be involved in ISGylation systems: HERC5, HHARI, and estrogen-responsive finger protein (EFP)/TRIM25. The majority of ISGylated proteins have been reported as targets of HERC5, while one, 4EHP, is the target of HHARI. TRIM25 has been reported to have more targets[18,19]. Protein ISGylation can be reversed by USP18 (UBP43), an enzyme that mediates the de-ISGylation, generating the free form of ISG15 known as "free ISG15"[20]. ISGylation alters protein–protein interactions, modifications, and activities.

UBIQUITINATION AND ISG15/ISGYLATION IN BREAST CANCER

The study of protein posttranslational modifications is critical for understanding the molecular basis of disease progression, such as carcinogenesis[21], and given the importance of ubiquitination in protein function, the number of reports in this field has increased rapidly in recent years. Ubiquitination is an important cellular process to study during cancer progression, as it is related not only to degradation by the proteasome, but also to the abundance, localization, activity, and interaction of proteins[22]. Several analyses have shown different roles for ubiquitination related to topology; for example, Lys-48-linked monoubiquitination is a signal for endocytosis and nuclear trafficking, but polyubiquitination at that site is a signal for proteasome-dependent degradation.

The UPS regulates many cellular processes, such as cell cycle control in breast cancer, where expression of cyclins (D1, E1) and cyclin-dependent kinases is altered, and their cellular role is directly correlated with the clinical aspects of breast cancer. Among the best-known cancer clinical markers with E3-Ub ligase activity are BRCA1 (breast cancer type 1 susceptibility protein) and TRIM25; BRCA1 is an important player in breast cancer, and one of its diverse functions is to mediate the formation of Lys-6-linked polyubiquitination as well as DNA damage repair[23].

TRIM25, the subject of this review, has been reported to be an important regulator in breast cancer. Regarding the role of TRIM25 in ubiquitination, several studies have shown its interaction with a number of proteins that are modified by Ub and degraded, as described in the next section. Ub ligases regulate a number of cellular

processes, including metabolism, homeostasis, cell cycle, stability of substrates and tumor suppressor and oncogenic activities. A high number of Ub ligases is deregulated in cancer as a consequence of altered posttranslational mechanisms, and this deregulation affects tumor induction, progression, and therapy resistance[21].

ISG15 and ISGylation are being studied in the context of breast cancer. It has been reported that the decrease in ISG15 or UBCH8 (an E2-conjugating enzyme for ISG15) by interfering RNA reduces proliferation, migration, and epithelial-mesenchymal transition in MDA-MB-231 breast cancer cells[24]. *ISG15* or *UBCH8* expression enhances the migration of breast cells, having a pro-tumor role. One mechanism proposed for ISGylation is interference with ubiquitination in breast cancer cell lines[24,25]. In contrast, *ISG15* displays antitumor activity *in vivo* since xenotransplantation of athymic mice with ISG15 knockdown ZR-75-1 or MDA-MB-231 breast cancer cells, resulted in an increase in tumor development and a minor recruitment of NK cells in comparison to controls. Furthermore, subcutaneous injection of recombinant purified free ISG15 near the xenotransplant of MDA-MB-231 cells in these mice, led to a decrease in tumor growth, increased NK cell infiltration in tumors, and enhancement of major histocompatibility complex surface expression[26]. Despite the need for further studies, the results suggest that protein ISGylation may be coupled to carcinogenic events and that extracellular free ISG15 is an "antitumor" potential candidate factor.

The proteins targeted by TRIM25 and modulated by ubiquitination or ISGylation as well as the specific effects of these processes on breast cancer progression are discussed below.

TRIM25: STRUCTURE AND FUNCTION

TRIM25 is a 17 beta-estradiol and type I IFN-inducible E3 Ligase, encoded by the *TRIM25* gene, which is located on the human chromosome 17q22. TRIM25 belongs to the TRIM family of proteins that is characterized by the presence of three conserved N-terminal domains: a RING domain, one or two B-Boxes (B1/B2), and a coiled-coil (CC) domain[27]. The RING domain has a catalytic activity, as it promotes ubiquitin conjugation by binding to a ubiquitin-conjugating enzyme (E2)[27,28]. B-box domains regulate TRIM oligomerization, and the CC domain is responsible for the dimerization of TRIM ligases. The CC domain is also responsible for and binding to RNA. Additionally, TRIM proteins possess a variable C-terminal region that mediates the interaction with substrates; specifically. TRIM25 has a PRY-SPRY or B30.2 domain, which is involved in protein-protein interactions and RNA binding[29] (Figure 2).

TRIM25 is involved in many cellular processes, such as development, innate antiviral immunity, and tumor progression[30]. Regarding its role in development, it has been reported that *TRIM25* is highly expressed in embryonic stem cells (ESCs)[31], and is an RNA-specific cofactor of Lin28a/TuT4-mediated uridylation[32] thus, it may be important for the maintenance of stemness[29]. Moreover, it is known that TRIM25 regulates estrogen-induced cell proliferation and uterine swelling, as mice that lack *TRIM25* have underdeveloped uterus and diminished estrogen responsiveness[33].

Another important function of TRIM25 is related to innate antiviral immunity. In that sense, it has been shown that TRIM25 polyubiquitinates the K63 residue of the cytosolic receptor RIG-1, promotes the interaction with mitochondrial antiviral signaling protein, and leads to the activation of intracellular antiviral signaling and subsequent type I interferon production[27].

Finally, for the purpose of this review, it is important to highlight the contribution of TRIM25 in tumor progression. It has been reported that TRIM25 is highly expressed in breast, colorectal, urothelial, and endometrial tumors, promoting proliferation, migration, and invasion of tumor cells[29,34]. These functions are regulated by different molecular mechanisms. First, it has been reported that TRIM25 is related to tumor growth, as it increases p53 Levels by inhibiting its ubiquitination and degradation by the 26S proteasomes[35]. Moreover, Takayama *et al*[36] have suggested that TRIM25 modulates p53 signaling by interacting with the androgen-induced GTPase-activating protein-binding protein 2 protein. Additionally, it has been reported that TRIM25 promotes cell survival and growth by directly targeting Keap1 for ubiquitination and degradation, leading to Nrf2 activation, which consequently activates antioxidant defense and ameliorates oxidative stress[37]. TRIM25 has also been shown to act as a transcriptional regulator of breast cancer metastasis networks[30]. For instance, TRIM25 regulates some critical stemness signatures, such as *POU5F1*, *SOX2*, and *NANOG*, which enhance cellular colonization.

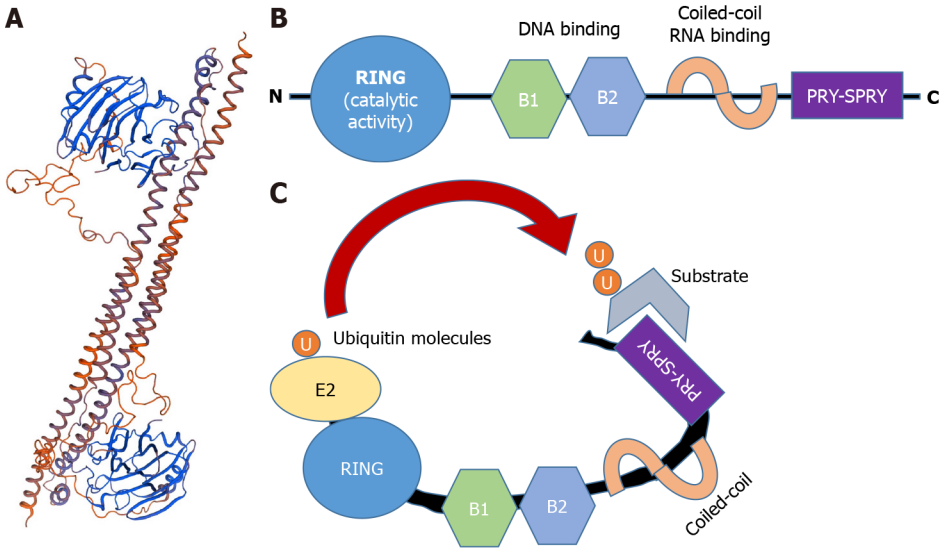


Figure 2 Structure of TRIM25. A: Crystal structure of the human TRIM25 coiled-coil and PRYSPRY domains. (DNA sequence retrieved from NCBI. Gene, https://www.ncbi.nlm.nih.gov/gene/7706#gene-expression 21/01/21, and 3D model created with Swiss Model: https://swissmodel.expasy.org/); B: Schematic representation of TRIM25, including the conserved RING, B boxes (B1 and B2), the coiled-coil domain and the C-terminal variable domain (CTD) PRY-SPRY; C: RING and CTD domains bind to the ubiquitin-loaded E2 and the substrate, respectively. Consequently, both molecules get closer, therefore favoring substrate ubiquitination.

The findings presented in the previous paragraphs demonstrate the importance of TRIM25 in various cellular processes, and how some of them are involved in the development and progression of cancer. Hence, TRIM25 has been proposed as a regulator for breast cancer, and therefore, a molecular target for the diagnosis, prognosis, and treatment of this disease[29,38,39].

REGULATION OF *TRIM25* EXPRESSION IN BREAST CANCER

The expression of *TRIM25* gene can be induced by IFN–type I a and b[13]. In addition, it has been reported that *TRIM25* has p53-responsive elements, inducing its expression in a p53–dependent manner upon DNA damage[40]. Among the most important stimuli that induce *TRIM25* expression in breast cancer cells are estrogens. The mechanisms related to the regulation of *TRIM25* expression are not fully understood. However, some of them have been investigated and the results indicate their complexity and possible association with several signaling pathways in several cellular contexts.

The human *TRIM25* gene covers approximately 25 kb of genomic DNA and is organized in nine exons (Figure 3A). TRIM25 is also named as EFP, since its expression in breast cancer cells is induced by estrogens within 30 min. At the mechanistic level, it has been demonstrated that the *TRIM25* 3´UTR has one estrogen responsive element (ERE). Interestingly, this region of *TRIM25* 3´UTR possesses an enhancer activity that acts in an estrogen-dependent manner, increasing TRIM25 expression in breast cancer cells[41]. Additionally, Urano *et al*[42] have reported that TRIM25 is an estrogen-responsive E3-Ub ligase. They found that implantation of transfected TRIM25-MCF7 cells into ovariectomized mice resulted in the formation of prominent tumors, while implantation of vector-transfected MCF7 cells did not result in the formation of tumors.

Furthermore, *TRIM25* expression can also be modulated by miRNA-3614-3p and proteins associated with mRNAs, such as IGF2BP3 (Figure 3B). The *TRIM25* 3´-UTR region contains the pri-miRNA-3614-3p, as well as a binding site for miRNA-3614-3p. Hence, miRNA-3614-3p prevents the expression of its own host gene, *TRIM25, via* miR-3614-mediated degradation, after its binding to the *TRIM25* 3´-UTR. Furthermore, IGF2BP3 protein can bind to the *TRIM25* 3´UTR to inhibit miRNA-3614 maturation. Breast cancer cell growth is blocked by *TRIM25* downregulation through two events: (1) miRNA-3614-3p overexpression or (2) IGF2BP3 depletion[43].

The regulation of *TRIM25* expression has an impact on other important proteins in breast cancer such as ERα. It has been demonstrated that ZBTB7A binds to regulatory

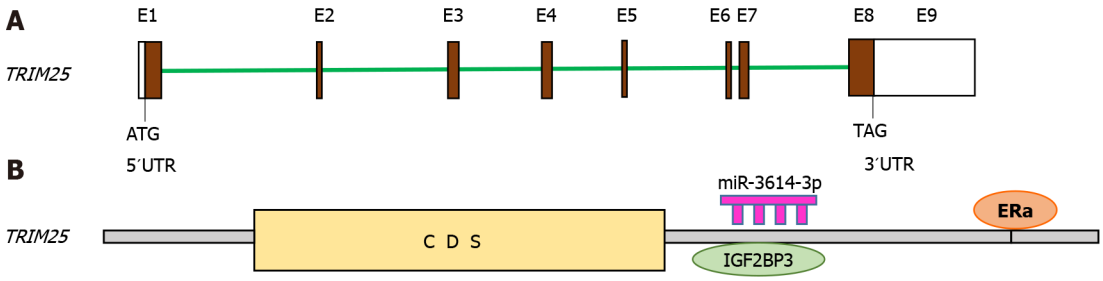


Figure 3 Structure of *TRIM25* gene and its regulation. The structure of the *TRIM25* gene is shown in (A) Regulation of *TRIM25* expression by ERα, (B) miRNA-3614-3p, and IGF2BP3 through its 3′UTR region.

regions of the gene that encodes for ERα (ESR1) to induce its expression in breast cancer cells, and regulates other ERα-dependent genes, facilitating breast cancer progression. Additionally, TRIM25 seems to be regulated by ZBTB7A since when ZBTB7A is inhibited, TRIM25 is upregulated. TRIM25 enhances ERα degradation *via* the UPS system, maintaining a positive correlation between ZBTB7A and ERα in breast cancer[44].

With respect to *TRIM25* expression in breast tissue, a study detected TRIM25 in the cytoplasm of normal breast tissue cells, and in cell culture media, as a secreted factor. Also, high levels of *TRIM25* have been detected in lactating breast, suggesting that TRIM25 may play a role in mammary gland differentiation. In a study low levels of TRIM25 were found by immunohistochemistry in breast cancer[45]. Nevertheless, in another study, TRIM25 immunoreactivity was positively correlated with poor prognosis of breast cancer[46].

TRIM25 AND PROTEIN UBIQUITINATION

Previous studies have shown that the Capicua (CIC) protein has an important role as a prognostic factor in different types of cancer, and this role is related to changes in the expression of targets downstream of the MAPK signaling cascade[47]. However, a MAPK-independent mechanism has been proposed where the interaction of CIC with ATXN1L prevents CIC degradation by TRIM25[48]. Whereas TRIM25 repression increases CIC levels and its tumor suppressor function, TRIM25 induction decreases it, indicating that CIC degradation is mediated by TRIM25 *via* the UPS. TRIM25 is enriched in both ATXN1L knockout and knockdown cell systems[48]. Another CIC interacting protein is 14-3-3 sigma, which has been shown to act as a tumor suppressor by negatively regulating the cell cycle and causing G2/M arrest. In breast cancer, 14-3-3 sigma expression is downregulated through epigenetic silencing by hypermethylation, and this inactivation has been suggested to be an early event in breast carcinogenesis[49]. A second mechanism for 14-3-3 sigma downregulation involves its interaction with TRIM25[42,50]. It has been shown that degradation of 14-3-3 sigma promoted breast tumor growth in a TRIM25 dose-dependent manner[42,50]. Overexpression of TRIM25 has been shown to drastically reduce the levels of 14-3-3 protein, which are recovered by MG132 (a potent inhibitor of proteasome function), suggesting that TRIM25 directly downregulates 14-3-3 sigma through a proteasome-dependent mechanism[42]. Several studies suggest that in human breast cancer, the regulation of 14-3-3 sigma may involve both mechanisms[49].

Another player in estrogen-dependent breast development and carcinogenesis is AT motif-binding factor 1 (ATBF1), which has been described as a potential tumor suppressor and is upregulated by direct binding of ERα to its promoter. TRIM25 also interacts with ATBF1, mediating its degradation by the UPS. Thus, ATBF1 antagonizes TRIM25-mediated cell proliferation[51]. It is also known that the transcription factor Kruppel-like factor 5 (KLF5), is a multifunctional transcription factor that inhibits estrogen-induced gene expression and cell proliferation. In breast cancer cells, estrogen-induced TRIM25 causes KLF5 protein degradation by the UPS. At the transcriptional level, estrogen downregulates KLF5, however, it takes a longer period of time than its effect at the protein level. It has been proposed that KLF5 and estrogen signaling regulate each other through a feedback mechanism, where high levels of KLF5 inhibit the function of estrogen by interacting with the ERα. Moreover, TRIM25 overexpression prevents KLF5 ubiquitination and enhances its own ubiquitination. Ubiquitinated TRIM25 is unable to interact with KLF5, whereas TRIM25 without

ubiquitination marks can associate with KLF5 in breast cancer cells[52].

TRIM25 MEDIATES ISGYLATION

Under DNA damage, a p53-dependent pathway induces 14-3-3 sigma expression, which sequesters cyclin B1-CDC2 complexes outside the nucleus, favoring G2 arrest. However, in breast cancer, the downregulation of 14-3-3 sigma is common due to CpG methylation, p53 inactivation, and degradation *via* the UPS. TRIM25 acts as an E3-Ub ligase, mediating 14-3-3 sigma degradation (as it was mentioned previously). Intriguingly, TRIM25 and the E2-Ub conjugating enzyme UBC8 participate in the post-translational modification of 14-3-3 sigma *via* ISGylation[53]. In addition to the identification of TRIM25 as an E3 Ligase for ISG15, it has also been shown that this activity is RING domain-dependent[19]. Interestingly, TRIM25 is a target of ISGylation through its own E3 ISG15 Ligase activity. The ring-finger domain of TRIM25 is implicated in its auto-ISGylation, and the TRIM25 Lysine modified by ISG15 is K117. TRIM25 carrying a mutation in this residue was resistant to its own ISGylation. However, this mutant EFP-K117R enhances the ISGylation of its target, 14-3-3 sigma. All data suggest that autoISGylation of TRIM25 inhibits its function as an ISG15 E3 Ligase and the ISGylation of its protein target, 14-3-3 sigma[54].

Moreover, the carboxyl terminus of Hsp70-interacting protein (CHIP) is an E3-Ub ligase that is a target of ISGylation in HEK293 cells upon stimulation with type I IFN. The ISGylation target residues of CHIP are Lys143/144/145 and Lys287, and as a result of this modification, the activity of CHIP as an E3 ubiquitin ligase is increased, affecting targets such as c-Myc. Hence, the ISGylation confers an antitumor activity to CHIP, and this modification can be mediated by HERC5 and TRIM25[55].

TRIM25 IN BREAST CANCER: FUTURE CHALLENGES

TRIM25 is a common component in the ubiquitination and ISGylation pathways. However, the mechanisms that define participation in one or the other system remain to be elucidated. Similarly, whether there is a competition between the TRIM25-dependent substrate ubiquitination or ISGylation should be evaluated. It is not completely known the effect of these modifications on the stability, localization, interaction profile, and the specific functions of their substrates in breast cancer. Furthermore, many other signaling pathways with cancer and anti-cancer activities may affect the expression and abundance of TRIM25 in mammary tumors.

TRIM25 is an ERα-regulated gene[41], and consequently, mammary tumors that do not express this receptor may have a different pattern of expression, regulation, and activity of TRIM25. In addition, the TRIM25-regulated proteome may be altered by treatment with anti-estrogens commonly used in endocrine therapy, such as tamoxifen (a type of SERM) and fulvestrant (a type of SERD). All these aspects related to TRIM25 may have impact in the detection, prognosis, and treatment of breast tumors. To date, the studies indicate that TRIM25 is a key element involved in molecular pathways that differentially modify the proteome in mammary tumors in comparison with normal mammary tissue.

TRIM25 is also involved in other cancer types. For example, it has been proposed that TRIM25 may be a biomarker and target for therapies against endometrial[56] and lung cancer[57]. Nevertheless, its ability to be induced in response to estrogens and its relationship with ERα-associated pathways indicate its relevance in breast cancer. Additional studies have shown that deregulation of TRIM25 is associated with metastasis and poor survival outcome of patients with breast cancer[30]. Moreover, it has been proposed that TRIM25 can modulate gene signatures at the transcriptional and post-transcriptional levels, in addition to its effect on the proteome in breast cancer. Therefore, the identification of proteins modified by TRIM25 may help to further establish its functions and relationships with other molecular mechanisms, and modifications in this disease.

CONCLUSION

In conclusion, the data presented here suggest that TRIM25 may be a biomarker in breast cancer. Although more studies are required to define the molecular mechanisms

of regulation and action of TRIM25, the investigations performed so far suggest that TRIM25 may be a potential target for new therapeutic strategies to control breast cancer progression.

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MINIREVIEWS

Current challenges in applying gene-driven therapies in clinical lung cancer practice

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Abstract

Over the last twenty years, with the development of gene-driven therapies, numerous new drugs have entered clinical use. Very few of these new drugs are suitable for a large number of patients, and all require molecular genetic testing. In lung cancer, gene-targeted therapy has evolved rapidly and has placed demands on the development of diagnostics and tissue sample preparation and logistics. Rapid diagnosis and prevalence assessment are necessary to determine the prognosis of a lung cancer patient based on the latest research findings. Therefore, the molecular-genetic diagnostic pathway must also be accelerated and matured to do the necessary analyses on small samples. Because lung cancer rebiopsy can be difficult, liquid biopsy techniques should be developed to cover more of the treatable mutations. There are obstacles related to tissue sampling, new genomic techniques and access to gene-driven cancer drugs, including their affordability. With this review and case study, we go into the obstacles faced by our clinic and discuss how to tackle these obstacles in lung cancer. We use lung cancer as an example due to its complexity, though these same obstacles are found in different cancers on a minor scale.

Key Words: Cancer drug; Availability; Gene testing; Tissue sampling; Lung cancer

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Core Tip: Several gene-driven therapy drugs and molecular testing, together with immunohistochemistry, have evolved for lung cancer in recent years. Lung cancer is mutation dense and has more predictive genes that potentially influence treatment decisions than any other cancer type. In our case study, we ran into obstacles and delays in the diagnostic pathway both in tissue sampling and in gene testing. However, the major obstacle was the financial availability of new drugs. All elements need to be in place before a new drug can be given to patients, following the idea of the right drug at the right time.

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INTRODUCTION

Since the sequencing of the whole human genome was completed in 2003[1], new principles of genomic approaches have eagerly been used in cancer drug therapy[2]. Early enthusiasm about genes in cancer drug therapy was overwhelming, and overall the outlook was positive. Gene-targeted drug development took new steps, and the first major drug was imatinib (Glivec). Imatinib served as a breakthrough by changing attitudes from one-size-fits-all concept to an outlook that focused on small, restricted indications. Ph-positive chronic myelogenous leukemia was found to inhibit the BCR-ABL1 tyrosine kinase with increased activity because of a definite genetic alteration [3], but given its high effectiveness, it rapidly dominated the whole set of indications and became an example of the importance of highly targeted drugs in restricted indications, achieving commercial success. After Glivec's success for limited indications, an increasing number of developers targeted limited indications with their therapeutic agents. The dominant thinking was that it would be better to have a highly active agent in limited indications than a less active one in a broader indication. It was important that the general attitude changed swiftly to allow specific approaches with gene-driven drugs.

Swift from cytotoxic chemotherapy agents towards targeted cancer drugs has been transpiring. By the end of 2014, the United States Food and Drug Administration (FDA) had approved 150 anticancer drugs, of which 89 were target-based and 61 were cytotoxic drugs[4]. Regarding solid cancers, drug agencies have become active in approving new agents based on solid early clinical data and scientific rationale. The FDA approved pembrolizumab in microsatellite-high tumors without any restrictions of cancer type[5]. Recently, both the FDA and the European Medicines Agency (EMA) approved larotrectinib, based on limited phase II data, for tumors with neurotrophic receptor tyrosine kinase (NTRK) gene fusion with all indications[6]. While these targeting agents add treatment possibilities, they also demand appropriate testing before their use.

Many targetable genes have emerged. Most new cancer drugs are linked to one gene mutation. Fortunately, molecular techniques have also been further developed, and gene tests have become more affordable. Now we have knowledge of multiple gene-driven cancers with certain gene alterations and the drugs that target them. Lung cancer is a prime example of a cancer with a wide variety of actionable mutations, partially because of the aggressive nature of the disease but also because of the existence of many mutations, some of which are recognized druggable driver mutations. Several gene-driven drugs are included in lung cancer guidelines. Even more drugs are in development, which require extensive clinical research before approval and implementation in clinical practice.

This article will address the practical obstacles in access to gene-driven cancer drugs in non-small-cell lung cancer (NSCLC). Small-cell lung cancer was not included due to high molecular heterogeneity and lack of current target genes with available treatment [7]. More than an approved cancer drug at the pharmacy is required. First, there need to be proper techniques for analysis; second, there must be enough tumor samples available; third, treatment procedures will need to allow the use of the drug; and finally, there needs to be funding for new drugs. We present the challenges involved

in using gene-driven therapies based on our clinical experience with lung cancer at a Finnish central hospital. Our practices are reviewed in the form of a case study.

Access to tumor samples and to treatment therapy procedures

Process of lung cancer treatment decision is complex and requires many elements as illustrated in Figure 1. Poor performance status, lung diseases and other comorbidities are patient-related factors that often make lung cancer diagnostics challenging. The patient's cooperation, sedation during tissue sampling and safe interruption of anticoagulation need to be considered during the planning phase of a safe procedure. Localized sampling of tissue consumes both time and resources, and both can be saved with good planning[8]. In lung cancer, sampling approaches vary based on the location and spread of disease. The most common sampling techniques are bronchoscopy, computed tomography (CT)-guided transthoracic lung biopsy and endosonography.

For mediastinal nodal staging in patients with suspected or proven NSCLC with abnormal mediastinal or hilar nodes on CT or positron emission tomography, endosonography is recommended over surgical staging as the initial procedure[9]. The three most common complications following endobronchial ultrasound with real-time guided transbronchial needle aspiration (EBUS-TBNA) are hemorrhage (0.6%), pneumothorax (0.4%) and infection (0.3%)[10]. Bronchoscopy and tissue sampling of intrabronchial cancer can be achieved in a combined session with EBUS-TBNA with proper sedation and cooperation. However, the tissue samples are small, most often cytological, materials, limiting the ability to extensively test them.

More material is available from core biopsies, but the risks are higher. The main complications in CT-guided transthoracic lung biopsy were pneumothorax (25% *vs* 19%), pulmonary hemorrhage (18% *vs* 6%) and hemoptysis (4% *vs* 2%) for core biopsy and fine needle aspiration (FNA), respectively. As shown by CT-guided core biopsy and FNA, the rate of pneumothorax requiring intervention was 6% and 4%, respectively[11]. Tissue sampling is an invasive procedure with risks, so the diagnostic pathway should be optimally planned to reduce the number of procedures needed to obtain enough material diagnosis and staging.

At the early stages, staging and tissue sampling require more advanced methods and time, whereas advanced metastatic cancer sampling is usually fast and easy. This might be one of the reasons why diagnosis time in lung cancer does not affect prognosis[12,13], while the stage of lung cancer at diagnosis does determine survival [14]. The faster pathway was shown to be beneficial in three studies[15-17]. In Denmark, improved diagnostic accuracy has led to significant improvements in survival rates and reduced mortality[18].

Access to new genomic techniques

It has been estimated that in the United States, approximately 80% of cancer patients are treated at community hospitals without access to advanced genomic testing[19]. Clinical implementations of advanced cancer genomics have been limited to academic cancer centers[19]. Advanced testing includes a combination of next-generation sequencing and computational data analysis. In lung cancer, targetable gene alterations are looked for first, including sensitizing epidermal growth factor receptor (EGFR) mutations, anaplastic lymphoma kinase (ALK) and ROS-1 rearrangements, the BRAF V600E mutation, NTRK 1/2/3 gene fusions, the METex14 skipping mutation and RET rearrangement[20]. However, in practice, the list may be shorter if there is no access to the cancer drug related to a given gene alterations. For example, in Finland it is common practice to sequence only EGFR, ALK, ROS-1 and NTRK, because their corresponding drugs have reimbursements available, and to leave others untested.

Keeling *et al*[21] defined common barriers to the clinical implementations of companion diagnostic tests. Specifically, if physicians are not aware of biomarkers, tests are not requested, and the quality and appropriateness of samples are essential because accurate analysis requires enough sample of good quality. Tests are not always available in existing laboratories. The results should be reliable and consistent. If physicians lose confidence in using a certain cancer drug, test results might not be available in time. Cancer treatment decision making usually requires fast test turnaround times; if the results take more than 2 wk, this will decrease the willingness to use the therapy. If tests are insufficiently reimbursed, then financial issues become an obstacle. All technical obstacles need to be covered before patients can be indicated for the rights treatment at the right time.

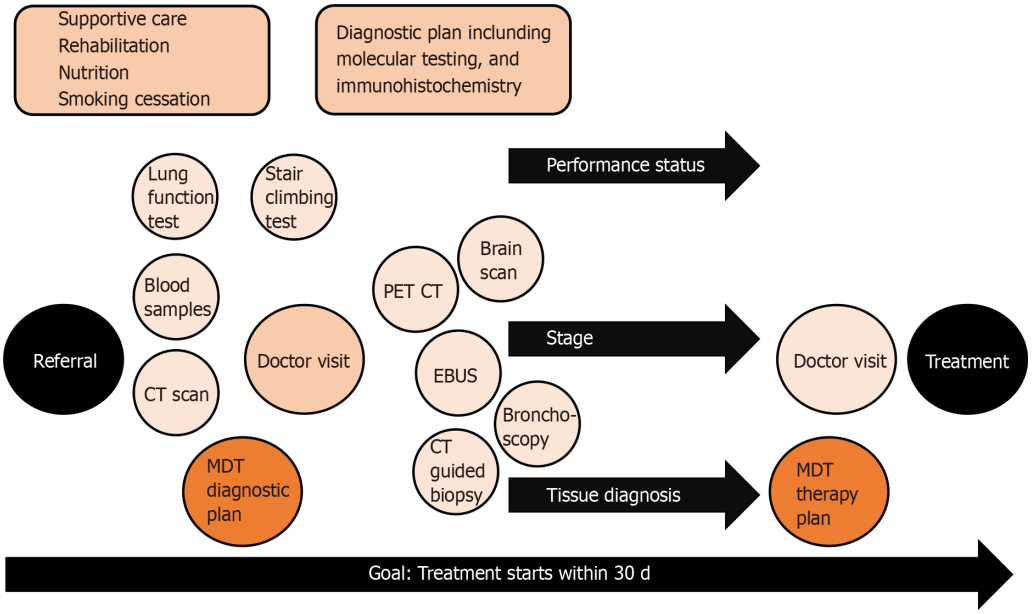


Figure 1 Process of treatment decision in lung cancer. After referral of patients' lung function tests, blood samples, computed tomography scans, and star climbing tests are reviewed in multidiscipline team (MDT) and the diagnostic plan formed, after which doctor's visit and defined examinations. Treatment decision including operation, radiotherapy and systemic therapy by MDT planning meeting is based on performance status, stage and histology. PET: Positron emission tomography; CT: Computed tomography; EBUS: Endobronchial ultrasound; MDT: Multidiscipline team.

Access to gene-driven cancer drugs

New drugs are entering clinical use *via* clinical trials, but not every country has access to the results of clinical trials. When a cancer drug is approved based on the results of clinical trials by drug registration agencies, it becomes available if the pharmaceutical company seeks authorization in a specific country. Usually, the FDA in the States is faster at approving than the EMA in Europe[22], but even faster approval might be done in other countries, such as Canada, Australia, Japan, or China. Although most applications rely on identical pivotal trials across regulatory agencies, only 57% of indications received either FDA or EMA approval and approval by an additional agency[23].

When a new cancer drug is approved, it becomes widely available, but there is an additional obstacle to its use, which is the price (Figure 2). All new cancer drugs, which are more expensive than old ones that are no longer covered by patents, should have an additional payer to at least partially support its high costs. Of course, the first payer is the patient, but support may come from insurance companies, government institutions or fraternal societies. Coverage varies widely. Every country has its own system based on available funds and evaluations that are performed mainly depending on efficacy results in clinical trials, as well as how the drug fits with treatments currently in use. Correspondingly, in Finland, coverage of cancer drug prices is in place. If a cancer drug is given in a hospital, it is provided by the hospital, which is receiving funding from neighboring counties and subsided by the government. If a cancer drug is taken at home in tablets, it may have governmental reimbursement. Price can be covered partially with a defined annual maximum for the patient, after which the drug is given free. Another possibility is full coverage with a nominal fee. If a tablet drug has no reimbursement, the patient needs to pay for it in full.

CASE STUDY –GENOMIC TESTING FOR LUNG CANCER IN THE VAASA ONCOLOGY CLINIC 2016-2019

The Vaasa Oncology Clinic covers 365000 people with approximately 50 annual first visit consultations for oncological treatment of lung cancer. Gene testing for all actionable mutations is implemented in our process. Altogether, 458 gene tests were analyzed during 2016-2019 (pyrosequencing 2016-2017 and then NGS 2017-2019), which covered 160 lung cancer patients (Table 1). Our focus was on EGFR driver gene mutations, as there has been a reimbursed cancer drug available for these patients. However, other actionable mutations, such as KRAS and BRAF mutations, were also

Table 1 Number of tested patients during years 2016-2019

	Pyrosequencing (all patients)	NGS(all patients)	Lung cancer	cfDNA	ALK (IHC)	ROS-1 (IHC)
2016	128	1	39	5	8	0
2017	82	29	38	10	6	0
2018	0	125	49	28	51	0
2019	0	93	33	12	34	16

NGS: Next generation sequencing; ALK: Anaplastic lymphoma kinase; IHC: Immunohistochemistry.

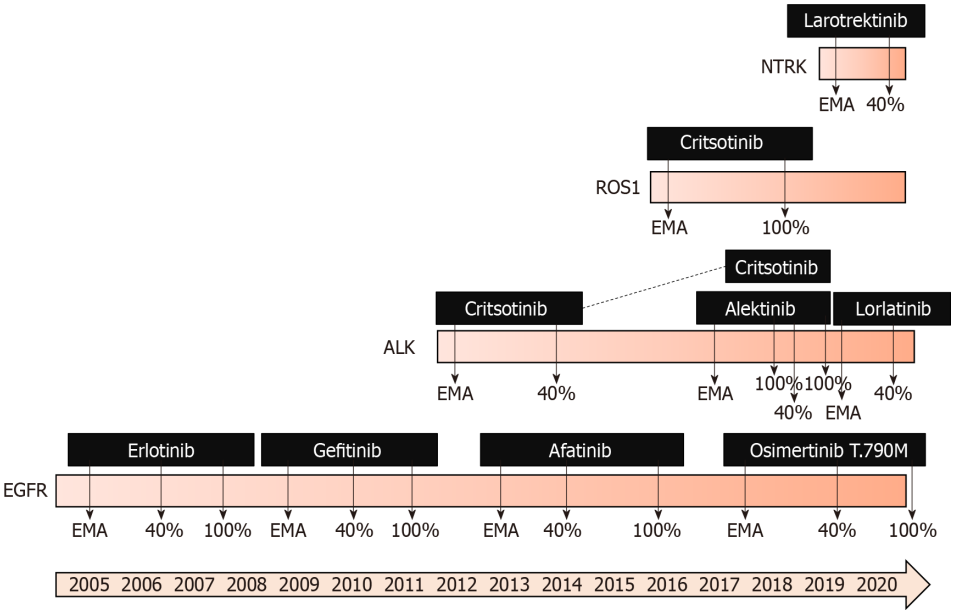


Figure 2 Presenting the history of the European Medicines Agency approvals and reimbursement situation in Finland (40% reimbursement and 100% reimbursement), concerning gene-driven therapies for lung cancer. NTRK: Neurotrophic receptor tyrosine kinase; PET: Positron emission tomography; CT: Computed tomography; EMA: European Medicines Agency; ALK: Anaplastic lymphoma kinase; EGFR: Epidermal growth factor receptor.

observed and reported. As the reimbursement situation was improved during the study years, ALK and ROS-1 genes were included in the testing pattern (immunohistochemically analyzed and positive results verified by FISH) in 2018 and 2019, respectively (Table 1). In addition to these tissue biopsy analyses, cfDNA liquid biopsy samples were analyzed by digital PCR targeting EGFR. All the gene analyses were conducted at the Jyväskylä Central Hospital Pathology laboratory in central Finland. To obtain the results from the implementation of the cancer treatment, the results were expected to be received within two weeks of time.

During 2016-2019, we found 16 EGFR-positive tumors from tissue biopsies (Table 2), corresponding to 5%-18% of tested patients/year (Table 2). The relative proportion of EGFR-positive patients was much higher during 2018-2019 (12% and 18%, respectively) than during 2016-2017 (approximately 5% in both years). This large difference can be explained in two ways: Either the patients were selected more precisely later on, or the sensitivity of the analytical method used to detect EGFR mutations was a determining factor (Table 1). In our protocol, all non-squamous-cell lung cancer patients who were considered for oncological treatment were tested, and the selection of the patients should not have been changed dramatically during the reported study years. cfDNA samples were studied to follow up the development of possible T790M resistant mutations, so of the patients were tested several times. The total proportion of cfDNA samples positive for EGFR mutations was 20%-25% (Table 2). Of all of these EGFR-positive patients, analysis based on paraffin-embedded samples and cfDNA samples guided 22 patients to EGFR-TKI therapies (afatinib, gefitinib and osimertinib). The other documented actionable mutations in lung cancer varied from 0% (ROS-1) to 30% (KRAS).

Table 2 Number of mutations found in genetic testing of lung cancer patients

	EGFR-pos	BRAF pos	KRAS pos	cfDNA EGFR-pos	ALK-pos	ROS-1-pos
2016	2 (5%)	NR	NR	1 (20%)	0	0
2017	2 (5%)	NR	NR	2 (20%)	0	0
2018	6 (12%)	1 (2%)	9 (18%)	6 (21%)	2 (4%)	0
2019	6 (18%)	2 (6%)	10 (30%)	3 (25%)	0	0

EGFR: Epidermal growth factor receptor; ALK: Anaplastic lymphoma kinase; NR: Not reported.

We are confident that we can identify lung cancer patients who would benefit from targeted therapies based on gene morphology. Initial biopsy and rebiopsy in lung cancer are challenging, but with an adequate team, safe sampling is possible. Early planning of the diagnostic procedure together with radiologists is advisable, and this daily interaction needs to be based on actual data. For fast processing, pathologists and molecular pathologists need to stay informed about the current requirements. Extensive testing takes almost two weeks at the moment, and this process needs to be made faster. Liquid biopsy as a follow-up tool for T790M development is working well.

DISCUSSION

Rapid diagnosis and prevalence assessment influence the prognosis of a lung cancer patient based on the latest research findings. Therefore, the molecular genetic diagnostic pathway must also be accelerated and developed to run the necessary tests on small samples. Because lung cancer rebiopsy can be difficult, liquid biopsy techniques should be developed to cover more treatable mutations. Even though it was relatively easy to find a laboratory in Finland that could do the required analyses by the proper techniques, our challenges were sampling and handling the samples in time in the laboratory. Rebiopsy for tissue samples was difficult to organize, partially because only restricted personnel could take the biopsies but also because sampling was often deemed too difficult or to have excess risks. Blood samples of liquid biopsy were far easier to take, but there we ran into the problem that analyses were less comprehensive when done on liquid biopsies than on paraffin samples. In fact, the only recognized reliable tests in liquid biopsy were for EGFR and its resistance mutation T790M, so those were the only ones done routinely. Logistics was also challenging, as our limit for clinical answers was 2 wk, which was a demanding timeline that was often exceeded by a couple of days. However, if gene results are not obtained in 2 wk, the treatment decision is jeopardized, and delays in the initiation of treatment were frequent. Similarly, gene-driven therapies would have fit nicely into our treatment procedure, but the biggest limitation was the lack of reimbursements. New gene-driven drugs were so expensive that ordinary Finns could not afford them. There needed to be a copayment, and there was not, the drug was not prescribed at all, and no gene testing was needed. Therefore, the reimbursement system effectively dictated cancer therapy. Finally, based on our experience, it was not the availability of gene testing, samples or procedures but rather national reimbursement for particular drugs that had the greatest impact on the use of gene-driven therapies.

In a recent OECD report, challenges in access to oncological medicines were addressed from a financial perspective[18]. Patients expect rapid access to new cancer drugs, but that is not the reality, mainly because of both clinical and financial uncertainties and the position of the new drug in relation to the standard therapies. Societal willingness and capacity to pay vary across countries, and health technology assessments face the difficulties of estimating cost effectiveness, determining the position of new drugs, and, in the case of multiple indications, dealing with populations of varying size. The rate of co-coverage ranges from free of charge to a copayment with or without a payment or coinsurance with or without a cap. The situation in Finland, which was not included in the report, is in the middle, with a fixed copayment for self-administered drugs and a fixed copayment without a cap for hospital inpatient care. Therefore, hospitals supported by small counties cover intravenous cancer drugs administered in outpatient wards, and a separate reimbur-

sement system is for prescribed tablet cancer drugs.

Similarly, we found in our case study that reimbursement was a major factor that actually influenced the use of gene-driven cancer drugs, as if there was no reimbursement, no testing was organized for the particular gene, and the cancer drug was not available for patients. Copayments are organized individually in each European country, and thus, there is heterogeneity in access to cancer drugs. Our Finnish system restricts considerable gene-driven cancer therapy. In our system, intravenous drugs are covered by hospitals and all tablets by copayment if approved for governmental reimbursement. Reimbursement decisions are slow in Finland and can take 4 years, similar to what happened with full crizotinib reimbursement (Figure 2). At the same time, marketing authorization comes fast after EMA approval.

CONCLUSION

The use of molecular testing and immunohistochemistry in lung cancer has evolved in recent years. We are still looking for the best combination of biomarkers, and negotiation among oncologists, invasive pulmonologists, invasive radiologists, lung pathologists and molecular pathologists continues. As the samples are small, both cytology and core biopsy, discussion what to prioritize is needed.

Lung cancer is mutation dense and has more predictive genes that potentially influence treatment decisions than any other cancer type. However, there are many obstacles to clear before a new drug becomes available for prescription by clinicians. The first requirement is the existence of a new cancer drug with efficacy relating to gene testing. Second, test methods and appropriate samples need to be available. Third, the new drug needs to be affordable to patients. All these elements need to be in place before the new drug is available to patients, following the concept of the right drug at the right time.

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MINIREVIEWS

Adjuvant therapy for lung neuroendocrine neoplasms

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Abstract

Pulmonary neuroendocrine neoplasms (NENs) represent a minority of lung cancers and vary from slower growing pulmonary carcinoid (PC) tumors to aggressive small cell lung cancer (SCLC). While SCLC can account for up to 15% of lung cancer, PCs are uncommon and represent about 2% of lung cancers. Surgical resection is the standard of care for early-stage PCs and should also be considered in early stage large cell neuroendocrine carcinoma (LCNEC) and SCLC. Adjuvant treatment is generally accepted for aggressive LCNEC and SCLC, however, less well established for PCs. Guidelines admit a lack of trials to support a high-level recommendation for adjuvant therapy. This manuscript will discuss the role for adjuvant therapy in NENs and review the available literature.

Key Words: Neuroendocrine; Adjuvant therapy; Lung; Pulmonary carcinoid; Small cell lung cancer

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Core Tip: Neuroendocrine neoplasms of the lung are uncommon malignancies. They vary in degrees of aggressiveness from the slow growing typical carcinoid tumors to the very fast-growing small cell lung cancer. While surgical resection should be considered for early-stage disease, the role of adjuvant therapy is less well established. Guidelines from different organizations vary, citing a lack of trials to support a high-level recommendation. This manuscript will discuss the evidence for and against adjuvant therapy in neuroendocrine neoplasms of the lung.

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INTRODUCTION

Neuroendocrine neoplasms (NENs) are heterogenous tumors derived from neuroendocrine cells, which are located throughout the body, including organs such as the thyroid, pancreas, genitourinary tract, gastrointestinal tract, and the lungs[1]. Most are found in the abdomen as gastrointestinal neuroendocrine tumors (50%-60%), but approximately 20%-30% are found in the lung[2]. Lung NENs are derived from the foregut and are thought to arise from specialized bronchopulmonary cells called Kulchitsky cells[1]. There are no known risk factors for low grade tumors; however, smoking is the major risk factor for high grade NENs[3].

Clinically, lung NENs behave very differently depending on grade and stage. The 2015 World Health Organization Classification assigns lung NENs into four categories: typical carcinoid (TC) low grade, atypical carcinoid (AC) intermediate grade, large cell neuroendocrine carcinoma (LCNEC), and small cell lung cancer (SCLC)[4]. These categories are based on mitotic rate, presence of necrosis, and cytological details. Specifically, TCs are defined as mitotic rate < 2 mitoses per high powered field (HPF) without necrosis, whereas ACs have a mitotic rate from 2-10/HPF with or without necrosis. The mitotic rate for both LCNEC and SCLC is > 10/HPF with extensive necrosis. The current staging follows the American Joint Committee on Cancer 8th edition for lung cancer[5]. Each category has a markedly different biological behavior with different prognostic features and treatments, making accurate diagnosis imperative[6].

Lung NENs generally present similarly to other lung malignancies with symptoms such as dyspnea, cough, or hemoptysis but can also be found incidentally as an asymptomatic pulmonary nodule or mass[7]. Multiphasic contrast-enhanced computed tomography (CT) is the gold standard of diagnostic imaging[8]. Functional imaging with ^{68}Ga dotatate PET/CT or ^{64}Cu dotatate PET/CT is recommended for TCs and ACs after pathologic confirmation following a biopsy[9]. ^{18}F-FDG PET/CTs are generally most useful for higher grade tumors along with an MRI of the brain[8]. If patients are found to be metastatic, treatment options for TC/AC include somatostatin analogs[10], everolimus[11], combination chemotherapy with capecitabine/temozolomide[12], and radionuclide therapy[13]. Treatment for metastatic high grade tumors generally involves chemotherapy along with immunotherapy[14-16].

The treatment for early stage TC and AC is primarily surgical resection with active surveillance post operatively[8,17]. Surgical resection generally follows non-small cell lung cancer guidelines with lobectomy and mediastinal node sampling being the standard of care, however, there may be a role for sub-lobar resections in low grade tumors. Adjuvant chemotherapy is generally accepted for patients with high grade disease, however, the role for adjuvant treatment in TC and AC patients is less well established. This manuscript will discuss the use of adjuvant treatment in patients with lung NENs.

MAIN

Adjuvant treatment in typical and atypical carcinoid tumors

The mainstay of treatment for TC and AC tumors of the lung is surgical resection. Complete resection offers excellent progression-free survival rates of 97% and 80% in TC and AC patients, respectively[18]. The utility of adjuvant treatment is not as clear. There is limited data on the effectiveness of adjuvant therapy in the literature and treatment beyond surgery is controversial. The rarity of this tumor makes prospective, randomized control trial difficult (if not impossible) to conduct, leading to a paucity of data examining the role of adjuvant therapy in this uncommon malignancy.

Guidelines from the National Comprehensive Center Network (NCCN), European Neuroendocrine Tumor Society (ENETS), and the European Society of Medical Oncology (ESMO) both state that surgical resection alone is recommended for TC and AC patients with stage I or stage II disease and TC with stage III tumors, without the utilization of adjuvant therapy in this setting[8,19,20]. The recommendation for Stage III AC tumors differs slightly between these societies. ENETS calls for the consideration of adjuvant treatment in stage III AC patients who harbor positive lymph nodes[19], whereas NCCN states that one may consider cytotoxic chemotherapy with cisplatin and etoposide, carboplatin and etoposide, or temozolomide in stage III AC tumors regardless of lymph node status (NCCN 2021). However, the NCCN goes on to state there is limited data of the efficacy of chemotherapy. ESMO advises that adjuvant treatment consisting of chemotherapy with or without radiation therapy may be considered in patients who have an especially high risk of relapse, such as AC N2 patients after multidisciplinary consultation[20]. All groups call for an individualized, patient centered approach and shared decision making with each patient. In 2020, the North American Neuroendocrine Tumor Society and the Commonwealth Neuroendocrine Tumor Research Collaboration published an update and an endorsement of the 2015 ENETS guidelines indicating adjuvant therapy with somatostatin analogs, chemotherapy or radiation, is not recommended in lung neuroendocrine tumors after complete resection because of the lack of data[10]. Please see Table 1 for a summary of the current recommendations.

The absence of decisiveness in the guidelines is due to a lack of available data; however, there are multiple studies that do not support the use of adjuvant treatment in TC and AC tumors. Presently, there are five large, retrospective studies that fail to demonstrate the effectiveness of adjuvant therapy (consisting of radiation therapy or chemotherapy such as cisplatin and etoposide, carboplatin and etoposide, or temozolomide) in these low and intermediate grade bronchopulmonary tumors. Three studies examined adjuvant treatment in AC and TC patients with stage I, II, and III disease[21-23], one study assessed adjuvant therapy in TC node positive disease[24], and the last study analyzed adjuvant treatment in AC patients[25]. Please see the conclusions of these studies outlined in Table 2.

Westin *et al*[23] aimed to determine the impact of adjuvant chemotherapy in patients with AC and TC who had node positive disease without evidence of distant metastasis. Of the 8240 TC and 8259 AC patients identified *via* the National Cancer Database (NCDB), 6% and 40% of patients received adjuvant treatment, respectively. Adjuvant treatment was associated with significantly worse outcomes in TC patients (HR: 3.8; P = 0.004) and no OS benefit in patients with AC (HR: 1.1; P = 0.6). The authors concluded that adjuvant treatment may be harmful in those with TC[23].

Similarly, Wegner *et al*[21] utilized the NCDB to identify 662 patients with stage I, II, or III TC or AC who had undergone surgical resection followed by adjuvant treatment consisting of radiation therapy, chemotherapy, or chemoradiation. In this study, adjuvant treatment was not associated with a survival benefit in any stage, with median overall survival (mOS) being 114 mo for adjuvant therapy *vs* 117 mo for observation (P = 0.30). Specifically, for stage III disease, mOS favored observation (79 mo *vs* 63 mo; P = 0.89) Thus, the study concluded that adjuvant treatment should not be routinely used in this population[21].

Additionally, Gosain *et al*[22] conducted a retrospective study using the NCDB to examine the use of adjuvant chemotherapy in TC and AC tumors. This group identified 6673 patients (88% TCs and 12% AC). There was no survival benefit in those with TC who received adjuvant chemotherapy at any stage and patients did well with surgery alone. Adjuvant treatment in stage I AC patients was shown to be harmful, with patients having a 5-year OS of 84% for observation *vs* 52% chemotherapy (P < 0.01). Stage II AC showed a trend towards worse outcomes with adjuvant therapy with 5-year survival of 81% for observation *vs* 55% for adjuvant therapy (P = 0.34), however, without statistical significance potentially owing to the few patients available for analysis. Stage III AC patients trended towards a benefit from adjuvant

Table 1 Summary of current available guidelines for the use of adjuvant therapy in the treatment of stage I, II, and III typical carcinoid and atypical carcinoid

Stage; Subgroup	Guidelines			
	CommNETS/NANETS	ENETS	NCCN	ESMO
I; TC	Surgery without AT	Surgery without AT	Surgery without AT	Surgery without AT
I; AC	Surgery without AT	Surgery without AT	Surgery without AT	Surgery without AT
II; TC	Surgery without AT	Surgery without AT	Surgery without AT	Surgery without AT
II; AC	Surgery without AT	Surgery without AT	Surgery without AT	Surgery without AT
III; TC	Surgery without AT	Surgery without AT	Surgery without AT	Surgery without AT
III; AC	Surgery without AT	Chemotherapy may be considered in patients with positive lymph nodes	Chemotherapy may be considered. RT is not recommended	Chemotherapy with or without radiation therapy may be considered in patients who are at high risk of relapse, (ex: N2 patients)

AT: Adjuvant therapy; AC: Atypical carcinoid; ENETS: European Neuroendocrine Tumor Society; ESMO: European Society of Medical Oncology; CommNETS: Commonwealth Neuroendocrine Tumour Research Collaboration, NANETS: North American Neuroendocrine Endocrine Tumor Society; NCCN: National Comprehensive Cancer Network; NR: No recommendations; TC: Typical carcinoid.

treatment (5-year OS was 46% for observation *vs* 54% for adjuvant therapy; $P = 0.24$); however, this also did not reach statistical significance[22]. As such, this study lends support to observation following surgery in stage I, II and III TC and stage I, II AC patients. Stage III AC patients had a trend towards improvement but without statistical significance[22].

One study aimed to determine the role for adjuvant therapy in TC alone. Nussbaum *et al*[24] identified 629 patients who had TC with node positive disease; of which, 37 patients (5.9%) were given adjuvant chemotherapy. Observation alone showed a significant survival advantage compared to adjuvant chemotherapy, with 5-year OS being 81.9% for observation *vs* 69.7% for adjuvant therapy ($P = 0.042$). However, after propensity matching, the statistical difference in OS was no longer observed, though a trend towards an increase in 5-year OS in the observation alone cohort was still present (80.9% observation *vs* 69.7% adjuvant therapy; $P = 0.096$). It was concluded by the authors that adjuvant chemotherapy did not improve overall survival among TC patients with node positive disease[24].

Anderson *et al*[25] aimed to determine the role for adjuvant chemotherapy in AC alone. This study identified 363 patients with node negative disease and 218 patients with node positive disease; of these patients, 15 and 89, respectively, received adjuvant chemotherapy. Among the 15 node negative patients who received chemotherapy, OS at 12 mo and 60 mo was 86.7% and 73.3%, respectively, compared to 87.9% and 72.3% among those being observed ($P = 0.54$). Among AC patients with node positive disease who had adjuvant treatment, survival at 12 and 60 mo was 98.9% and 47.9%, respectively, compared with 98.4% and 67.1% survival at 12 and 60 mo, respectively, in those who underwent observation ($P = 0.46$). This study concluded that there was no survival advantage for adjuvant chemotherapy among those with node positive or negative AC[25].

In contrast, three studies lend support for the consideration of therapy after surgery in TC and AC patients[22,26,27]. Unlike the five aforementioned studies that do not support the use of adjuvant therapy, these three studies have a small number of participants, consisting of two single institution retrospective studies[22,26] and one case report[27]. The conclusions of these studies are outlined in Table 2.

Herde *et al*[26] conducted a single institution study describing their use of adjuvant treatment, consisting of either chemoradiation or radiation alone, in 47 TC and 12 AC patients from 1989 to 2009. Of the 59 patients, 55 had surgery and 8 received adjuvant therapy. There were 5 patients (1 TC and 4 AC), who received chemoradiation, 1 AC patient who was node positive received radiation, and 2 AC patients who were found to have metastasis received palliative radiation and palliative chemotherapy. This group found that reserving adjuvant treatment for TC and AC patients with adverse pathologic features was an effective strategy for their patient population. This study reported a 20% recurrence rate among these patients with unfavorable pathological features, which is lower than what has been reported in the literature. Thomas *et al*[28] report that 63.6% of AC patients develop metastasis at a median time of 17 mo after

Table 2 Summary of the available studies examining the use of adjuvant therapy in the treatment of stage I, II, and III typical carcinoid and atypical carcinoid

Study information; Stage and Subgroup	Ref.							
	Westin *et al* [23], 2017	Wegner *et al* [21], 2019	Gosain *et al* [22], 2019	Nussbaum *et al*[24], 2015	Anderson *et al*[25], 2017	Herde *et al* [26], 2018	Chong *et al* [27], 2014	Buonerba *et al*[29], 2010
AT	Chemotherapy	Chemotherapy, radiation or chemoradiation	Chemotherapy	Chemotherapy	Chemotherapy	Chemoradiation or radiation alone	Chemoradiation	Chemotherapy; SSA
Study	Retrospective; NCDB	Retrospective; NCDB	Retrospective; NCDB	Retrospective; NCDB	Retrospective; NCDB	Retrospective; Single Institution	Retrospective; Single Institution	Case Report
Subgroup	TC/AC	TC/AC	TC/AC	TC	AC	TC/AC	TC/AC	AC
Stage	IIB,III	I,II,III	I,II,III	IIB,III	I,II,III	I,II,III	IIB,IIIA	I
Other	node +	NA	NA	node +	Comparing node + and node -	NA	NA	NA
I; TC	NA	Surgery without AT[3]	Surgery without AT	NA	NA	AT in patients with adverse pathologic features[9]	NA	NA
I; AC	NA	Surgery without AT[3]	Surgery without AT[@] (5-yr OS of 84% in obs *vs* 52% AT; $P < 0.01$)	NA	Surgery without AT in node - (OS at 12 and 60 mo in AT 86.7% and 73.3%, *vs* obs 87.9% and 72.3% $P = 0.54$).	AT in patients with adverse pathologic features[9]	NA	Chemotherapy followed by SSA; 10-yr PFS
II; TC	Surgery without AT (inferior OS with AT)[1]	Surgery without AT[3]	Surgery without AT	Surgery without AT[5,6] (5-yr OS 81.9% obs, *vs* 69.7% AT; $P = 0.042$)	NA	AT in patients with adverse pathologic features[9]	chemotherapy may be beneficial in a subset of patients[8]	NA
II; AC	Surgery without AT (no OS benefit)[2]	Surgery without AT[3]	Surgery without AT (5-yr survival of 81% in obs *vs* 55% AT; $P = 0.34$).	NA	Surgery without AT in node +[7]	AT in patients with adverse pathologic features[9]	chemotherapy may be beneficial in a subset of patients[8]	NA
III; TC	Surgery without AT (inferior OS with AT)[1]	Surgery without AT[4]	Surgery without AT	Surgery without AT[5,6] (5-yr OS 81.9% obs, *vs* 69.7% AT; $P = 0.042$)	NA	AT in patients with adverse pathologic features[9]	chemotherapy may be beneficial in a subset of patients[8]	NA
III; AC	Surgery without AT (no OS benefit)[2]	Surgery without AT[4]	Surgery without AT but trend towards benefit (46% in obs *vs* 54% AT; $P = 0.24$)	NA	Surgery without AT in node +[7]	AT in patients with adverse pathologic features[9]	chemotherapy may be beneficial in a subset of patients[8]	NA

[1]Stage IIB, III grouped together and was associated with inferior OS (HR: 3.8; 95%CI: 1.9-7.0; $P = 0.004$).
[2]Stage IIB, III grouped together with no befit in OS (HR: 1.1; 95%CI: 0.68-1.78; $P = 0.6$).
[3]TC and AC stage I and II tumors grouped together to show a median survival of 114 months versus 117 months with observation ($P = 0.30$).
[4]TC and AC stage III tumors favored observation with a median survival of 79 mo *vs* 63 mo ($P = 0.89$).
[5]Adjuvant treatment showed a harmful effect.
[6]When baseline patient features were matched a statistical difference in OS no longer existed but there was a towards detriment (69.7% *vs* 80.9%; $P = 0.096$).
[7]Node + stage II and III grouped together; OS at 12 and 60 mo in AT 98.9% and 47.9%, *vs* obs 98.4% and 67.1%; $P = 0.46$.
[8]Stage IIB and IIIA grouped together; at 2-yr follow up, there was a 28% recurrence rate for those with local regional disease.
[9]Stage I, II, III AC and TC grouped together; overall AT showed a 20% recurrence rate.
AT: Adjuvant therapy; AC: Atypical carcinoid; Obs: Observation; OS: Overall survival; NA: Not applicable; NCDB: National cancer database; PFS: Progression free survival; SSA: Somatostatin analogue; TC: Typical carcinoid.

diagnosis. As such, the authors conclude that adjuvant therapy may be considered in

In a similar study, Chong *et al*[27] conducted a retrospective analysis in their institution between 1990 and 2004 to help elucidate the role of adjuvant treatment in both TC and AC patients. There were 220 TC and 80 AC patients identified who underwent surgery, of which 7 patients with local regional disease (1 patient with TC and 6 with AC; 6 stage IIIA and 1 stage IIB) received adjuvant therapy with platinum and etoposide chemotherapy in combination with radiation. At 2 year follow up, there was a 28% (n = 2) recurrence rate for those with local regional disease. The authors state that for a subset of patients with TC and AC, chemotherapy may be beneficial [27].

Lastly, a case report of a women with surgically resected AC tumor who underwent 6 cycles of carboplatin, etoposide, and epirubicin followed by a somatostatin analog for 10 years demonstrated no evidence of recurrence[29]. Of importance, the tumor was staged as a IB; based on the initial five studies discussed, this patient most likely would have done well without chemotherapy. The major limitation of these three studies is the size of their patient population. While their contribution to the literature is very important, it is difficult to draw sound conclusions from studies with such small numbers.

Taken together, these eight studies indicated that there is no convincing evidence to support the use of adjuvant chemotherapy in stage I, II or III TC and stage I or II AC. Additionally, survival may be compromised if adjuvant therapy is given to this population. With respect to stage III AC, survival benefit was not demonstrated in the literature, despite one large study demonstrating a trend to improved overall survival. Larger, prospective studies are necessary to further consider the role of adjuvant treatment in pulmonary carcinoid tumors. Until then, we caution the utilization of adjuvant treatment in stage I, II, III TC or AC patients.

Small cell lung cancer

Small cell lung cancer (SCLC) has rapid doubling time and is rarely diagnosed at early stages when upfront surgical resection is possible. It is important to rule out occult metastasis with appropriate imaging like CT of the chest, abdomen, and pelvis; PET/CT or bone scans; and MRI of the brain since delaying systemic chemotherapy in patients with metastatic disease can have detrimental outcome. The patients who are surgical candidates require pathologic mediastinal staging to confirm staging. Patients who are then confirmed to have limited stage small cell lung cancer (LS-SCLC), without hilar or mediastinal nodal involvement (TNM stages I-IIA), may be considered for resection of the primary tumor with mediastinal lymph node sampling or dissection. Based on historical phase II clinical trials and observational studies, NCCN and ESMO guidelines recommend that patients without lymph node involvement, confirmed on surgery, receive adjuvant chemotherapy alone and those with unforeseen lymph node involvement should be considered for systemic chemoradiotherapy[30-33]. An analysis of National Cancer Database between 2003-2011 showed that the patients who received adjuvant chemotherapy ± radiotherapy had five-year survival of 53% compared to 40% in those who underwent surgery alone [34]. Other retrospective studies have shown a survival benefit of adjuvant chemotherapy, however, these studies have not been able to show a reduction in recurrence rates[32,35]. Concurrent chemoradiation is preferred over alternating sequential chemotherapy and radiation treatment in the adjuvant setting based on extrapolation of studies among non-surgical LS-SCLC patients showing numeric improvement in survival[36].

Cisplatin-based adjuvant chemotherapy, particularly Etoposide plus cisplatin (EP) for 4 cycles, has been the preferred regimen since 1990s after being shown to have a superior response rates, survival benefit, and improved toxicity profile over conventional alkylator or anthracycline based regimens[37-39]. The EP dosage regimens that has been studied with concurrent or sequential chemoradiotherapy include etoposide 100 mg/m^2 on days 1–3 and cisplatin 75 mg/m^2 on day 1, or etoposide 100 mg/m^2 and cisplatin 25 mg/m^2 on days 1–3 or etoposide 120 mg/m^2 on days 1-3 and cisplatin 60 mg/m^2 on day 1[40,41]. Each cycle is for 21-28 d.

Systematic analysis has shown similar efficacy of carboplatin compared to cisplatin-based regimens. In an analysis of four clinical trials with 663 patients (only 32% with LS-SCLC), comparatively similar number of patients received cisplatin- and carboplatin-based regimens and the outcomes were compared[42]. For the entire cohort, median progression-free survival (mPFS) was 5.5 and 5.3 mo (HR: 1.10; P = 0.25), and median OS was 9.6 and 9.4 mo for cisplatin and carboplatin groups, respectively (HR: 1.08; P = 0.37). As expected, the toxicity profile was different between the groups. However, it must be noted that since the intent is curative in the adjuvant setting and with the absence of prospective trials, carboplatin is only

recommended in patients who have contraindications for the use of cisplatin. The use of myeloid growth factors are also not recommended during concurrent chemoradiotherapy due to the increased risk of thrombocytopenia (and not due to risk of pulmonary toxicity)[43].

The addition of paclitaxel to EP in concurrent chemoradiation regimens has been evaluated in LS-SCLC. However, there is lack of data in the adjuvant setting, and although higher response rates were achieved with the addition of paclitaxel, there was substantial toxicity and lack of significant survival benefit[44,45]. Studies on the use of other agents including antiangiogenic agents and immunotherapy are limited to the non-adjuvant LS-SCLC or ES-SCLC setting.

A highly selected group of patients may be considered for surgical resection after induction chemotherapy, but there is limited data supporting this approach. A phase III randomized controlled trial included 340 patients with LS-SCLC who received 5 cycles of cyclophosphamide, vincristine, and doxorubicin and were determined suitable for resection on restaging[46]. These patients were randomized to thoracic radiation plus prophylactic cranial irradiation with or without surgery. The response rate to chemotherapy was 66%. Median survival was 15.4 mo in the surgical arm compared to 18.6 mo in the non-surgical arm and was not statistically significant (P = 0.78). A smaller phase II trial, however, showed better outcomes with surgery after induction chemotherapy ± radiation[47]. Eight patients with stage IB-IIA underwent 4 cycles of EP and 32 with stage IIB-IIIB received 3 cycles of EP plus thoracic radiation. Both of the groups underwent restaging and 23 underwent complete resection. The pathologic complete response was 34% at surgery. Patients who had an R0 resection had an overall survival advantage over unresected/not completely resected patients (mOS 68 mo *vs* 13 mo; P = 0.01). Similar findings were evident in a retrospective study of 75 patients with stage I-IIIA treated with 3 cycles of EP, 46 of which underwent thoracotomy and 35 had resection done[48]. Four additional cycles of EP were given after surgery. The pathologic complete response rate was 16% and these patient experienced tumor-free survivals of 136+ mo. Among patients who underwent resection, median survival of the cN0-1 subset was 25.09 mo and for cN2 it was 13.75 mo.

Large cell neuroendocrine carcinoma of the lung

With most of the data on adjuvant chemotherapy for LCNEC extrapolated from SCLC trials and other retrospective studies, it is conventionally treated similar to SCLC. It should be noted that large cell carcinoma (LCC) without neuroendocrine differentiation, lacking adenosquamous features, as well as immunohistochemical findings of SCLC, is a diagnosis of exclusion. Electron microscopic and molecular analysis of LCC show that the majority of these cases share features of non-small cell lung cancer and are managed accordingly[4,49].

A nonrandomized prospective study by Iyoda *et al*[50] included 15 patients with LCNEC (Stages I-IV) who received adjuvant chemotherapy with cisplatin 80 mg/m^2 on day 1 and etoposide 100 mg/m^2 on days 1-3 for 2 cycles. Recurrence rate was significantly lower in the adjuvant chemotherapy group compared to historic control (13.3% *vs* 60.87%, P = 0.0065). There was only one death from disease recurrence in the adjuvant group on 5-year follow up. Three patients had grade 4 toxicity, and 10 patients had grade 3 hematologic or gastrointestinal toxicity during treatment.

In a single center retrospective analysis done between 1992 to 2008 with 77 LCNEC and 23 combined LCNEC patients, 24 received induction chemotherapy and 25 adjuvant chemotherapy[51]. Twenty-two of the induction chemotherapy patients received platinum-based combination therapy and 2 received non-platinum single agent chemotherapy. Six patients received etoposide. 68.2% of the patients who received platinum-based combination therapy displayed a partial response, and 31.8% had stable disease. Of the 2 patients who received nonplatinum chemotherapy, 1 had progression of disease and 1 had stable disease. Among the adjuvant chemotherapy group, 20 received adjuvant platinum, and 15 of these received etoposide. Fifteen patients also received adjuvant radiation. 42% of patients received both induction and adjuvant chemotherapy. Stage IA had a 5-year OS of 72% and stage IB 26% (P = 0.0006). The 5-year OS for T1 disease was 66%, T2 44%, T3/4 30%, N0/1 56%, N2/3 24%. There was no association between OS and receipt of induction or adjuvant platinum-based chemotherapy. The 5-year OS was 50% for patients who did not receive chemotherapy compared to 45% for patients who received platinum-based chemotherapy (P = 0.18). However, when restricted to patients with Stage IB-IIIA disease with R0 resection, the 5-year OS was better in patients who received platinum-based chemotherapy as induction or adjuvant treatment compared to those who received other treatments (mOS 7.4 years *vs* 2 years, P = 0.052) with 5-year OS reaching

51% in the platinum-chemotherapy group.

Irinotecan and platinum chemotherapy have also been studied in the adjuvant setting and have not been shown to be superior to EP. The recent Japanese phase III trial among 221 stage I high grade NEC patients (39 patients with SCLC, 38 LCNEC, 14 combined LCNEC and 20 combined SCLC) randomized to EP and IP showed similar PFS[52]. In the LCNEC/combined LCNEC subset, 3-year relapse-free survival was 66.5% for EP *vs* 72.0% for IP (HR = 1.072; 95%CI: 0.517-2.22). Grade 3/4 adverse events were more frequent among the cisplatin-etoposide arm (20% *vs* 4%). There is no available data on other chemoimmunotherapy agents as adjuvant treatment in LCNEC and may be considered in the setting of clinical trials only.

CONCLUSION

Unlike non-small cell lung cancer where adjuvant therapy is well established, adjuvant therapy for NENs of the lung continues to lack prospective trials that definitively guide best treatment options. It is generally well accepted that high grade NENs of the lung (SCLC, LCNEC) that are completely resected should undergo adjuvant therapy. The role of adjuvant therapy in the lower grade TCs and ACs is less well accepted and multiple studies show a detrimental effect and should be avoided outside of a clinical trial. This represents an unmet need in the treatment for NENs of the lung, and prospective studies are warranted.

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ORIGINAL ARTICLE

Retrospective Study

Macrometastasis at selective lymph node biopsy: A practical going-for-the-one clinical scoring system to personalize decision making

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Author contributions: Herrero M, Ciérvide R, Calle-Purón ME, Valero J, Buelga P, Rodriguez-Bertos Isabel, Benassi L and Montero A conceived the clinical protocol; Herrero M, Buelga P, Rodriguez-Bertos I and Benassi L carried out the surgical procedures; Valero J encouraged Mercedes Herrero to investigate this topic and supervised the findings of this work; Herrero M analyzed data; Calle-Purón ME verified the analytical methods; Ciérvide R and Montero A wrote the manuscript, tables and figure; All the authors read, reviewed and accepted manuscript.

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Abstract

BACKGROUND

Axillary sentinel lymph node biopsy (SLNB) is standard treatment for patients with clinically and pathological negative lymph nodes. However, the role of completion axillary lymph node dissection (cALND) following positive sentinel lymph node biopsy (SLNB) is debated.

AIM

To identify a subgroup of women with high axillary tumor burden undergoing SLNB in whom cALND can be safely omitted in order to reduce the risk of long-term complications and create a Preoperative Clinical Risk Index (PCRI) that helps us in our clinical practice to optimize the selection of these patients.

METHODS

Patients with positive SLNB who underwent a cALND were included in this study. Univariate and multivariate analysis of prognostic and predictive factors were used to create a PCRI for safely omitting cALND.

RESULTS

From May 2007 to April 2014, we performed 1140 SLN biopsies, of which 125 were positive for tumor and justified to practice a posterior cALND. Pathologic findings at SLNB were micrometastases (mic) in 29 cases (23.4%) and macrometastasis (MAC) in 95 cases (76.6%). On univariate analysis of the 95 patients with MAC, statistically significant factors included: age, grade, phenotype, histology,

16.04.0940-GHM. And having evaluated the project from an ethical and methodological point of view, it issues a FAVORABLE OPINION for the project to be carried out by the main researcher Dr. Mercedes Herrero Conde, from the Gynecology service of HM Hospitales.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data sharing statement: The datasets generated and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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lymphovascular invasion, lymph-node tumor size, and number of positive SLN. On multivariate analysis, only lymph-node tumor size (≤ 20 mm) and number of positive SLN (> 1) retained significance. A numerical tool was created giving each of the parameters a value to predict preoperatively which patients would not benefit from cALND. Patients with a PCRI ≤ 15 has low probability (< 10%) of having additional lymph node involvement, a PRCI between 15-17.6 has a probability of 43%, and the probability increases to 69% in patients with a PCRI > 17.6.

CONCLUSION

The PCRI seems to be a useful tool to prospectively estimate the risk of nodal involvement after positive SLN and to identify those patients who could omit cALND. Further prospective studies are necessary to validate PCRI clinical generalization.

Key Words: Sentinel lymph node biopsy; Complete axillary lymph node dissection; Preoperative clinical risk index; Macrometastasis

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Core Tip: The role of completion axillary lymph node dissection (cALND) following a positive sentinel lymph node biopsy (SLNB) is being actively debated. Patients with a positive SLNB performed at our institution who also underwent a cALND were analyzed. Univariate and multivariate analysis of prognostic and predictive factors were used to create a Preoperative Clinical Risk Index (PCRI). The PCRI could help estimate the risk in removing extra positive nodes beyond the SLN in order to identify which patients could safely avoid cALND. Further prospective studies are necessary to validate clinical generalization for suggested tool.

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INTRODUCTION

The presence of axillary nodal involvement continues to be a major breast cancer prognostic factor. Complete axillary lymph node dissection (cALND) has a dual role both to ensure local control of the disease and to add staging information to adapt adjuvant treatment. The extent of cALND has evolved over time. In 1990, consensus from National Institute of Health concluded that the local treatment of axillary disease in operable breast cancer included at least the removal of axillary levels I and II[1]. In many European countries, cALND included all three Berg levels[2].

Axillary recurrence after cALND is infrequent[3-6], although treatment-related morbidity could be relevant both in the short- and long-term, including lymphedema, which occurs in 23 to 25% of women[7-11], pain, and decreased mobility of the arm[10,12]. During the 1980s, the possibility to identify a regional lymph node that was the first lymph node drainage station for breast cancer began to be considered[13]. Years later, different studies established the utility of selective sentinel lymph node biopsy (SLNB) as an alternative to axillary dissection in patients with clinically negative lymph nodes (cN0)[14-16], with the advantage of decreasing the risk of lymphedema associated with cALND[9,10,17].

The presence of sentinel node involvement was associated with the need to complete the study and treatment of the axilla with cALND. In recent years, emphasis has been placed on the possibility of reducing the need of cALND in patients with positive SLNB without worsening the prognosis. The results of the ACOSOG Z0011 [18] and IBCSG 23-01[19] trials suggest that cALND could be avoided when sentinel

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node is minimally involved, eliminating complications of axillary surgery without negatively affecting survival. In addition, the AMAROS[7] and OTOASOR[20] trials concluded that axillary radiation therapy was not inferior to cALND in those patients with clinically node-negative breast cancer who had a positive SLN. However, doubts remain regarding macroscopic involvement after SLNB. Aforementioned studies show wide variability regarding tumor burden in the sentinel node, including patients with isolated tumor cells (ITC), micrometastasis and macrometastasis making it impossible to definitively establish the effect of omitting cALND in patients with a higher tumor burden in the SLNB.

Our objective was to identify a subgroup of women with high axillary tumor burden in SLNB in whom cALND can be safely omitted in order to reduce the risk of long-term complications. The second endpoint is to create a Preoperative Clinical Risk Index (PCRI) that helps us in our clinical practice to optimize the selection of these patients.

MATERIALS AND METHODS

We retrospectively reviewed the experience of our Breast Cancer Unit with SLNB and cALND procedures from 2007 to 2014. This study received ethical approval from the Inhouse Local Ethics and Clinical Committee (Code: 16.04.0940-GHM) the April 27, 2016.

Inclusion criteria

All patients included in this analysis had breast cancer diagnosed by core needle biopsy. Axillar presurgical ultrasound with fine needle biopsy was done if required. Magnetic resonance imaging (MRI) of the breast was performed in all patients where it was technically feasible. Before 2012, SLNB was only done in patients with tumors less than 3 cm. However, since then, tumors smaller than 5 cm were also considered for SLNB. The sentinel lymph node assessment was performed intraoperatively, with hematoxylin-eosin and immunohistochemistry analyses deferred. Data from all patients with positive SLNB who underwent cALND were collected. From 2012, cALND was not carried out in the presence of micrometastasis.

Database information included the patients' age, menopausal status, and laterality, as well as tumor characteristics at diagnosis (tumor size, number of foci, IHC, Her-2 status, grade, presence of intraductal carcinoma), surgical treatment data (surgery type, date, margins status, postoperative tumor size), and data of lymph nodes analysis (SLN, nodes from the accessory axillary fat and nodes from axillary dissection). In each of the sections, isolated tumor cells (ITC), micrometastasis (mic) and macrometastasis (MAC) were differentiated.

All patients with pathologic involvement of the axilla received adjuvant whole breast/chest wall radiotherapy together with comprehensive regional nodal irradiation (RNI) according to published international guidelines[21,22].

Statistical analysis

Patients´ information was obtained from the patients´ records, and collected data were subsequently analyzed with the SPSS software, version 17.0 (Released 2008. IBM SPSS Statistics for Windows. Version 17.0 Armonk, NY: IBM Corp).

Once the data had been validated and refined, the statistical analysis was carried out following this way: (1) Exploratory analysis of all selected variables; (2) Descriptive analysis of the sample: distribution of all variables; (3) Description of the clinical data: patients, tumor and type of surgery; (4) Description of sentinel node biopsy procedures (detailed pathological results of SLNs, axillary fat and axillary dissections); (5) Descriptive statistics of the patients with macrometastasis in the sentinel node; (6) Univariate analysis with the Chi-square test was performed to compare every clinical parameter with the variable "presence" or "absence" of positive nodes in the lymphadenectomy". The parameters considered in the univariate analysis of the 95 cases of MAC were: age, menopausal status, laterality, tumor size, uni- or multicentricity, grade, phenotype, histology, association to in situ carcinoma, type of surgery, number of removed nodes, number of MAC, and the sum of MAC and mic; (7) For those significant variables in the univariate analysis, the Odds Ratio were calculated by using a logistic regression model using the Stepwise method to know which of them had the greatest impact on final results. The Pearson correlation coefficient was used. The level of significance used was $\alpha = 0.05$ (95% confidence interval), therefore those differences whose P value < 0.05 were considered significant;

and (8) Finally, a numeric value was assigned to selected variables by calculating the logarithms of the results to develop a numerical tool, the PCRI. Different cut-off points of this tool, according to the cut-off point recommended by Krag *et al*[14] in the NSABP 32 trial, were simulated in order to find the one that left the least number of false negatives (low risk, FN must be < 10%, specificity of 90%).

RESULTS

From May 2007 to April 2014 a total of 1140 women underwent an SLNB procedure. One-hundred and twenty-five (11%) were positive for tumor and, hence, underwent posterior cALND. The average number of sentinel nodes dissected was 2.12 (1-5). Pathologic findings after SLNB were micrometastasis (mic) in 29 cases (23.4%) and macrometastasis (MAC) in 95 cases (76,6%).

We focused our analysis on patients with MAC. In our patients, the probability of harboring additional lymph node disease in the cALND in patients with MAC in SLNB was 36%.

Patients' characteristic of the 95 patients with SLN macrometastasis are summarized in Table 1.

Univariate analysis

For the 95 patients with MAC, the parameters that showed statistical significance in the univariate analysis were: Age (cut point 40 years-old), grade, phenotype, histology, lymphovascular invasion, tumoral size, and number of positive sentinel nodes (P < 0.05 for all comparisons) (Table 2).

Multivariate analysis

When analyzing all the parameters that had been significant in the univariate analysis, only the tumor size less or equal to 15 mm and the presence of 1 affected SLN remained significant. The variable of up to 2 sentinel lymph nodes affected by MAC or mic as suggested by ACOSOG Z0011 was also studied. In our population, the presence of one MAC is the variable with the greatest statistical significance for predicting disease in the axillary dissection (Table 3). Of the 95 patients collected with MAC, 79 cases had only 1 affected SLN. For this reason, this data has been used as a cut-off point for the population analyzed.

PCRI

To avoid bias in the results due to the simultaneous presence of mic, a subgroup of 79 patients with exclusively one sentinel node with MAC in SLNB was selected from our original population, which served as the basis for developing the PCRI tool. Although having such a group of cases with little probability of involvement of other nodes in the cALND, these patients form a heterogeneous group. Thus, in the 79 women with only 1 affected SLN, we still found an additional 30.4% of disease in cALND, which is not an acceptable result to justify stopping cALND in all patients.

Based on the results of the univariate logistic regression of those variables that are related to the presence of additional disease on cALND (size, age, histology, grade and phenotype) in the group of 79 cases with presence of only one macrometastatic SLN, a numerical tool was created by giving each of the parameters a numeric value in order to be able to predict preoperatively which patients would not benefit from a cALND (Table 4). To facilitate the usability of the prognostic index, we decided to include only those factors that could be known before breast surgery. Appropriate numeric value was calculated by the resulting rate of involvement when considering this value in isolation and then taking these results to base 10 (Figure 1).

Numeric values assigned to age, grade, size, histological type, and phenotype were included. The resulting value of the PCRI was related to the risk of having other lymph nodes involved in the axillary dissection. According to the PCRI, patients could be divided into two groups based upon a cut-off point of 15.

Patients in the low-risk group (index score ≤ 15, 42 patients) were at risk of having other affected nodes less than 10% and in whom the cALND would have added to morbidity with minimal extra information. Only 5% changed from the pN1a state after axillary surgery. Ninety-five percent of patients had 1 or 2 positive nodes in total.

Patients in the high risk group (index score > 15, 37 patients) had a 54% of risk of having nodal involvement in the cALND. The pN1a stage changed in 13.5% of patients after lymphadenectomy. Seventy-three percent of the patients had 1 or 2 positive nodes in total, and 13.5% had 3 affected lymph nodes. For these patients, axillary

Table 1 Characteristics of the 95 patients with sentinel lymph nodes with macrometastases

Characteristics	*n* = 95	%
Age (range), yr	51.9 (23-82)	-
Average tumor size, mm	17.4	-
Tumor size		
0-10	14	14.7
11-15	25	26.3
16-20	26	27.4
21-30	18	18.9
31-40	6	6.3
> 50	1	1.1
In situ	5	5.3
Menopausal status		
Pre	50	52.6
Post	45	47.4
Histology type		
Ductal	76	80
Lobular	15	15.8
Others	4	4.2
Phenotype		
Luminal	83	87.4
Basal	9	9.5
Her-2	3	3.2
Grade		
I	16	16.8
II	54	56.8
I + II	70	73.7
III	24	25.3
Unknown	1	1.1
Surgery		
Conservative	63	66.3
Mastectomy	32	33.7
Number of SN removed		
1	35	36.8
2	28	29.5
3	20	21.1
≥ 4	12	12.6
Nº of positive SN (Mic + MAC)		
0	4	4.2
1	65	68.4
2	20	21.1
3	4	4.2
≥ 4	2	2.1

Size of the SLN metastasis		
ITC (i+)	0	0
Micrometastasis (Mic)	0	0
Macrometastasis (MAC)	95	100
Number of positive SLN		
0	55	57.9
1	21	22.1
2-3	11	11.6
1-3	32	33.7
≥ 4	8	8.4
Adjuvant treatments		
Radiotherapy	75/90	82.4
Chemotherapy	72/90	80
Hormonotherapy	79/90	87.7
Immunotherapy	4/92	4.3

SLN: Sentinel lymph nodes.

dissection would be indicated, not only as information for adjuvant treatment, but also as a therapeutic approach of the axillary disease itself.

Of the total of 79 cases with only one MAC detected by SLNB, using the cut-off point at 15 for the PCRI, less than 10% of the cases would have more affected nodes, and only 5% of the cases would change from the pN1a stage.

DISCUSSION

The American Society for Medical Oncology (ASCO) and National Comprehensive Cancer Network (NCCN) initially proposed in their clinical guidelines to always perform cALND in patients with positive SLNs[23,24]. However, studies had shown that 53% of patients with positive SLN did not have disease in the non-sentinel nodes after cALND[25], and that positive SLN was the only metastasis in 40% to 60% of cases [14,26-29]. In about half of the women undergoing cALND, surgery would not provide more prognostic information, although it would add a greater risk of complications derived from the procedure. Therefore, the current recommendations of international Guidelines and Consensus Statements on the locoregional treatment of breast cancer state the possibility of omitting cALND in the case of a positive SLNB is considered low risk, especially in those patients who are going to receive radiotherapy after surgery[24,30]. Omission of cALND is more frequently offered to patients who present lymph node involvement in the form of micrometastasis exclusively than in those patients with macrometastasis in the SLN[30-32].

When this study was conceived and designed, 2 large randomized trials had demonstrated the efficacy and safety of omitting cALND in select groups of patients[7, 33]. Compared with what was observed by ACOSOG Z0011[18] and AMAROS[7] trials, our study presents very similar characteristics, both in the mean age and in the rate of ductal or lobular tumors, although in our series we found 8% more grade I/II tumors and half the cases of lymphovascular invasion (LVI) than reported in ACOSOG Z0011, which is considered a marker of aggressiveness and worse prognosis[34]. Finally, it should be noted that the tumor phenotype is known in all of our patients including hormone receptors, overexpression of the HER2 protein and the proliferation factor Ki-67. These data were not collected in the AMAROS, however, the ACOSOG Z0011 study collected data on hormone receptors, but not on Her2 or Ki 67, making a direct comparison difficult. With respect to the surgery performed, in ACOSOG Z0011, all patients received conservative surgery. In AMAROS, the number of conservative surgeries drop to 82%. In our study, 70% of the patients had conservative surgery while 30% received mastectomies. This could allow us to apply the

Table 2 Univariate analysis of 95 patients with presence of sentinel lymph nodes with macrometastases

	No additional metastatic lymph nodes	Additional metastatic lymph nodes	*P* value
Age			
< 40	66%	44%	0.04
≥ 40	32.6%	67.4%	
Hormonal status			
Premenopausal	70%	30%	NS
Postmenopausal	60%	40%	
T stage			
T1	75.8%	24.2%	< 0.05
T2	47%	53%	
T3	0	100%	
Tumor size			
≤ 15 mm	85%	15%	< 0.05
> 15 mm	49%	51%	
Histologic type			
Ductal carcinoma	67.1%	32.9%	NS
Lobular carcinoma	47%	53%	
Grade			
I + II	68.6%	31.4%	NS
III	54%	46%	
Molecular subtype			
Luminal A/B	65.6%	34.4%	< 0.05
Basal	22.2%	77.8%	
HER2	100%	0	
LVI			
Present	66.7%	33.3%	NS
Absent	61.5%	38.5%	
Number of positive SLN			
1	69.6%	31.4%	< 0.05
≥ 2	43.7%	66.3%	
ACOSOG Z0011 criteria			
≤ 2 SLN+	68.5%	31.5%	< 0.05
> 2 SLN+	16%	84%	

LVI: Lymphovascular invasion; NS: Not significant; SLN: Sentinel lymph nodes.

study's conclusions regardless of the type of surgery. The results of the SLNB and cALND are very similar in our study to those reported by AMAROS and ACOSOG Z0011. In our study, 69% of patients did not have additional metastatic involvement in cALND, similar to 72% and 73% of AMAROS and ACOSOG Z0011, respectively.

Our study seeks to identify which patients will not have more axillary disease, even though the SLNB is positive for macrometastasis. The presence of macrometastasis in SLNB has been related to a higher probability of finding additional metastatic lymph nodes after cALND. According to other groups' experience, the incidence of involvement in the cALND beyond the SLN was from 40 to 58%[23] when in the presence of MAC, while for mic it fell to 20% (23), and for ITC (isolated tumor cells), it

Table 3 Multivariate analysis of 95 patients with sentinel lymph nodes with macrometastases

		P value	Odds ratio
Tumor size	≤ 15 mm *vs* > 15 mm	0.002	2.004
Number of metastatic SLN	1 *vs* > 1	0.021	2.573
ACOSOG Z0011 criteria	≤ 2 SLN+ *vs* > 2 SLN+	0.045	10.195

SLN: Sentinel lymph nodes.

Table 4 Univariate analysis of variables related to the presence of additional disease on completion axillary lymph node dissection in the group of 79 cases with presence of only one macrometastatic sentinel lymph nodes

	Additional metastatic lymph nodes, *n* = 79
Age	
< 40	50%
≥ 40	28%
≤ 15 mm	85%
> 15 mm	49%
Histologic type	
Ductal carcinoma	28%
Lobular carcinoma	50%
Mucinous	0
Tumor size	
≤ 10 mm	7%
11-15 mm	10%
16-20 mm	42%
> 20 mm	47%
Grade	
DCIS	0
I	18%
II	31%
III	45%
Molecular subtype	
Luminal A/B	28%
Basal	55%
HER2	0

was roughly 12% (23). In our series, the probability of harboring additional lymph node disease in the cALND in patients with MAC in SLNB was 36%.

Currently, there are 3 ongoing trials to provide evidence in this regard. The SINODAR ONE trial[35] randomized patients with 1 or 2 macrometastasis in SLNB and treated with conservative surgery or mastectomy to receive either cALND remain under observation. Both groups received adjuvant therapy. The INSEMA trial[36] included patients with tumors T1-2 and conservative surgery or mastectomy and were randomized to undergo either SLNB remain under observation. Patients with a positive SLNB with 1 or 2 affected nodes are then re-randomized to exclusive SLNB or cALND. Finally, in the POSNOC trial[37], T1-2 breast carcinomas with 1 or 2 macrometastasis in SLNB are randomized after conservative surgery or mastectomy to either undergo axillary treatment with cALND or RNI or remain under observation. In both arms the indicated systemic treatment is added. The results of these 3 studies

Risk index (PCRI) Preoperative Clinical Risk Index (PRCI) ≤ 15/> 15				
Age	**≥ 40**	**< 40**		
	2.8	**5**		
Histological type	**Ductal**	**Lobular**		
	2.8	**5**		
Phenotype	**Her2**	**Luminal**	**Basal**	
	0	**2.8**	**5**	
Grade	***In situ***	**GI**	**GII**	**GIII**
	0	**2**	**3**	**5**
Tumor size (mm)	**≤ 10**	**11-15**	**16-20**	**> 20**
	0	**1**	**4**	**5**

Figure 1 Numeric values assigned to each variable to create an individualized Preoperative Clinical Risk Index.

might elucidate. the feasibility and safety of omitting cALND in patients with macrometastasis in SLNB.

Attempts have been made to develop tools to estimate the risk of finding additional positive lymph nodes beyond the SLN based on the presence of different factors: multifocality, presence of LVI, or the hormone receptors status. To date, there are currently seven published nomograms in use: the Memorial Sloan Kettering Cancer Center nomogram[38], the Cambridge nomogram[39], the Turkish Federation nomogram[40], the Stanford nomogram[41], the MD Anderson Cancer Center nomogram[42], the Tenon nomogram[43], and the MOU nomogram[44]. However, these nomograms do not seem to have good reproducibility in populations other than their own[45]. For a nomogram to be successful and clinically applicable, it must identify the majority of patients who might have non-sentinel node involvement, and the false negative rate should be ≤ 10%[45]. We have analyzed prognostic factors in our treated patients in order to design a personalized tool that, preoperatively and based on clinical and tumoral data, would help us to select patients with macrometastasis determined by SLNB with a risk of less than 10% of additional positive nodes in cALND, which would potentially allow them to omit this procedure regardless of the type of surgery and adjuvant treatments, similar to the initial approach of Sentinel Lymph Node Biopsy in NSABP. Our clinical index differs from other widely distributed nomograms, such as the Memorial Sloan Kettering Cancer Center nomogram (MSKCCn) and the MD Anderson Cancer Center nomogram (MDACCn) designed to infer involvement beyond the SLNB. One factor that is different in our clinical index is that our index takes into account the age of the patient, which is not included in either the MSKCCn or the MDACCn. However, tumor size is assessed identically in both the MSKCCn and the MDACCn, as well as in our PCRI. Histology and grade are assessed jointly in the MSKCCn, while MDACCn does not assess tumor grade. Both nomograms assess the total number of nodes removed: MSKCCn as negative sentinel nodes, and MDACCn as total nodes removed. The presence of multifocality was associated with a higher risk in the MSKCCn, but not in our index, and it was not assessed in the MDACCn. LVI is recorded in both nomograms, since it implies a worse prognosis. Our data support this result, but as it is not a parameter that we can obtain preoperatively, and therefore was not included in our index. Likewise, the extranodal extension included in the MDACCn is not a parameter that we can have preoperatively, so it has also not been included in our index. Finally, the tumor phenotype is not collected in the MDACCn and is only partially collected in the MSKCCn, which assesses the absence or presence of estrogen receptor in the tumor.

The most important strength is that our PCRI is that the information can be obtained preoperatively, allowing a personalized decision-making process that involves the patient before surgery. The biggest weakness of our study is the retrospective nature of the study, which limits the findings of the study. The population we assessed does not correspond to a specific geographic area, nor has participated in a standard screening program, making the findings less biased for a specific population. Our

patients come both from our own gynecology consultations, where opportunistic screening is performed following the American guidelines[23], as well as from other centers. However, the patients have private health insurance policies that could cause selection bias from the population. Additionally, comparing the characteristics of our population with the multicenter studies, American Z0011 and European AMAROS, we did not find any significant differences between the three patient populations. In addition, the phenotypes of basal and Her-2 were not as represented as other phenotypes in the total of patients, suggesting those patients should be specifically discussed in the Tumor Board.

CONCLUSION

More than half of our patients with the presence of MAC in the SLNB had no involvement of the non-sentinel nodes, and only 1 in 8 changed from the pN1a stage, which means that we have performed too many cALND on our patients.

The PCRI we outlined is based on a detailed analysis of the group of patients with MAC in the SLN, avoiding the biases of mic common in other trials, and is a useful tool to prospectively estimate the risk of nodal involvement beyond the positive SLN and to identify those patients who would benefit from a cALND afterwards.

Further studies are necessary to validate feasibility and accuracy of this PCRI.

ARTICLE HIGHLIGHTS

Research background

The role of completion axillary lymph node dissection (cALND) following positive sentinel lymph node biopsy (SLNB) is being actively debated.

Research motivation

Patients with positive SLNB performed at our institution who underwent a cALND were analyzed.

Research objectives

This study aims to create a Preoperative Clinical Risk Index (PCRI).

Research methods

Univariate and multivariate analysis of prognostic and predictive factors were used to create a PCRI.

Research results

PCRI could help estimate the risk of having extra positive nodes beyond the SLN in order to identify in which patients cALND could be safely omitted.

Research conclusions

Further prospective studies are necessary to validate clinical generalization for the suggested PCRI.

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ORIGINAL ARTICLE

Retrospective Study

Gastric cancer molecular classification based on immunohistochemistry and in situ hybridization: Analysis in western patients after curative-intent surgery

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Abstract

BACKGROUND

Gastric cancer (GC) is a highly heterogeneous disease, and the identification of molecular subtyping of gastric adenocarcinoma emerged as a promising option to define therapeutic strategies and prognostic subgroups. However, the costs and technical complexity of molecular methodologies remains an obstacle to its adoption, and their clinical significance by other approaches needs further evidence.

AIM

To evaluate the clinicopathological characteristics and long-term survival of GC based on the subgroups of molecular classification by immunohistochemistry (IHC) and in situ hybridization (ISH).

METHODS

We retrospectively evaluated all patients who underwent D2-gastrectomy between 2009 and 2016 in a Western cohort of GC patients treated with curative intent. Microsatellite instability (MSI) status, E-cadherin, and p53 expression were

approved by the hospital ethics committee and registered online (https://plataformabrasil.saude.gov.br), No. CAAE: 37009120.0.0000.0068.

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analyzed by IHC, and Epstein-Barr virus (EBV) by ISH. Tissue microarrays were constructed for analysis. Clinicopathological characteristics and survival of GC were evaluated according to subtypes defined by The Cancer Genome Atlas (TCGA) Research Network Group and Asian Cancer Research Group (ACRG) classification systems.

RESULTS

A total of 287 GC patients were included. Based on IHC and ISH analysis, five profiles were defined as follows: E-cadherin aberrant (9.1%), MSI (20.9%), p53 aberrant (36.6%), EBV positivity (10.5%), and p53 normal (31%), which corresponded to tumors that showed no alteration in another profile. A flowchart according to the TCGA and ACRG classifications were used to define the subtypes, where clinical and pathological characteristics associated with GC subtypes were evidenced. Proximal location ($P < 0.001$), total gastrectomy ($P = 0.001$), and intense inflammatory infiltrate ($P < 0.001$) were characteristics related to EBV subtype. MSI subtype was predominantly associated with advanced age ($P = 0.017$) and the presence of comorbidities ($P = 0.011$). While *Laurén* diffuse type ($P < 0.001$) and advanced stage ($P = 0.029$) were related to genomically stable (GS) subtype. GS tumors and microsatellite stable/epithelial to mesenchymal transition phenotype subtype had worse disease-free survival (DFS) and overall survival (OS) than other subtypes. Conversely, MSI subtype of GC had better survival in both classifications. Type of gastrectomy, pT and the TCGA subtypes were independent factors associated to DFS and OS.

CONCLUSION

The IHC/ISH analysis was able to distinguish immunophenotypic groups of GC with distinct characteristics and prognosis, resembling the subtypes of the molecular classifications. Accordingly, this method of classification may represent a viable option for use in a clinical setting.

Key Words: Stomach neoplasms; Gastric cancer; Translational medical research; Molecular classification; Immunohistochemistry; Biomarkers

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Core Tip: In this study, patients with gastric cancer (GC) were retrospectively evaluated based on subgroups of molecular classification by immunohistochemistry and in situ hybridization. Microsatellite instability status, e-cadherin, p53 expression, and Epstein-Barr virus were evaluated in a Western cohort of GC patients treated with curative intent, where it was possible to obtain subgroups with different clinicopathological characteristics and prognosis. Thus, our findings demonstrate that through techniques used in the routine pathological evaluation it is possible to identify immunophenotypic groups of GC similar to those determined by the molecular classification.

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INTRODUCTION

Gastric cancer (GC) remains one of the most common cancers and the 3rd cause of cancer-related mortality globally[1]. In addition to this important burden on public health, GC management also represents a challenge due to its great heterogeneity in presentation and behavior. Tumors that appear to have similar characteristics may have extremely different outcomes. In order to better characterize GC, different classi-

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fications using clinical and histological aspects have been employed[2-4]. However, these parameters are not able to contemplate the whole disease spectrum.

Recently, classifications based on the molecular profile of GC were proposed[5,6]. The major molecular classification systems published by The Cancer Genome Atlas (TCGA) and by the Asian Cancer Research Group (ACRG) established several subtypes of GC related to distinct clinical and molecular characteristics, providing a molecular subtyping structure, as well as a guide to target agents.

Through complex analyses of molecular data, a TCGA study identified four distinct molecular subtypes: Epstein-Barr virus (EBV)-positive; microsatellite instability (MSI); genomically stable (GS); and chromosomal instability (CIN)[5]. Meanwhile, the ACRG proposed a different classification based on tumor protein p53 (TP53) status, constituted by the subtypes: MSI, microsatellite stable/epithelial to mesenchymal transition (MSS/EMT), MSS/TP53+, and MSS/TP53-[6].

Despite some differences, molecular classifications can be defined by four major signatures: EBV-positivity, alteration of mismatch repair (MMR) proteins, *TP53* gene, and cellular cohesion. Although the current molecular classifications have expanded our insight regarding the complex nature of GC, the technologies used to achieve such knowledge have not yet reached full clinical utility. The cost and complexity remain significant obstacles to translate these methodologies into daily clinical practice.

To overcome the complexity of molecular analysis, the search for other methods that could reproduce those findings led to the emergence of different strategies to define molecular subtypes. Techniques such as immunohistochemistry (IHC) and in situ hybridization (ISH) were employed successfully to define the molecular subtypes[7-9]. Indeed, the assessments of EBV status by ISH and MSI by immunostaining for DNA MMR proteins (MutL homolog 1, MLH1; MutS homolog 2, MSH2; MutS homolog 6, MSH6; PMS1 homolog 2, PMS2) are already well-defined methods in diagnostic practice. With regard to the other GC subtypes, p53 staining can be a simple and feasible method to detect gene alterations indirectly, common in the CIN and MSS/TP53+ subtypes. While e-cadherin staining is easily assessable, and the loss of staining is associated with diffuse-type histology and mesenchymal-like morphologic features, which are characteristic of the GS and MSS/EMT subtypes[5-10].

Thus, the aim of this study was (1) to define GC subtypes based on MSI, E-cadherin, and p53 expression determined by IHC and EBV status by ISH; and (2) to evaluate the clinicopathological characteristics and survival of these subtypes according to TCGA and ACRG classification in a western cohort of GC patients treated with curative intent.

MATERIALS AND METHODS

Patients

This study included all patients with GC who underwent potentially curative gastrectomy with lymphadenectomy identified between 2009 and 2016 from a prospective database. We included patients with gastric adenocarcinoma, D2 lymphadenectomy, and R0 resection. Gastric stump tumors, metastatic, palliative resections, and patients with formalin-fixed paraffin-embedded tissue (FFPE) blocks unavailable for analysis were excluded.

Abdominal and pelvis computed tomography, endoscopy, and laboratory tests were used for preoperative staging. Tumor stage was classified according to the 8th edition of the TNM[11]. Chemotherapy (CMT) was indicated for locally advanced GC (N+ and/or T3-4) with potentially resectable tumor. Lymph node (LN) dissection was performed based on the guidelines of the Japanese GC Association[3].

Postoperative follow-up was performed every 3 mo in the first year-and every 6 mo in the following years. Follow-up exams for recurrence detection were performed based on the presence of symptoms. Clinical outcomes were evaluated, including disease-free survival (DFS) and overall survival (OS). The study was approved by the hospital ethics committee and registered online (https://plataformabrasil.saude.gov.br/; CAAE: 37009120.0.0000.0068).

Pathological analysis

Hematoxylin and eosin (HE)-stained histologic sections from all patients included in the study were reviewed to tissue microarray (TMA) construction. Representative tumor areas (3 tumor and 2 adjacent non-tumoral mucosa tissue cores) were selected from each case, and punched out from individual FFPE tumor blocks using a precision mechanized system. TMA blocks were sectioned at 4 μm thick, and histological

sections were submitted to HE staining, IHC and ISH.

For IHC reactions, all sections were de-paraffinized in xylene and rehydrated through graded ethanol. IHC to MSI status was performed with Ventana automated staining system (BenchMark Ultra), according to the manufacturer's instructions. The following monoclonal antibodies were used for MMR proteins: Anti-MLH1 (clone M1), anti-MSH2 (clone G219-1129), anti-MSH6 (clone-44), anti-PMS2 (clone EPR 3947), all ready to use. The reactions were qualitatively interpreted according to the deposition of the chromogen product on the nuclei of the cancer cells. GC was considered negative only if there was a complete absence of tumor cells staining. GCs negative for MLH1, MSH2, PMS2, or MSH6 expression were considered deficient for MMR (dMMR) proteins, constituting the MSI profile. Tumors that maintained the expressions of all markers were defined as microsatellite stable (MSS status).

For E-cadherin (clone 36B5) and p53 (clone DO-7) staining, peroxidase activity was blocked with hydrogen peroxide and antigen retrieved by heat induction using citrate buffer (pH 6.0) Then, slides were incubated with the primary antibody (4 °C). Avidin-biotin free short polymer-based peroxidase amplification system and diaminobenzydine as chromogen were used for development of reaction products. After the reactions, sections were counterstained with hematoxylin.

A scoring system was used for the evaluation of E-cadherin expression. Scores 0 and 1 (0 = complete loss; and 1 = cytoplasmic expression) were considered aberrant expression. Scores 2 and 3 were defined as normal status (2 = cytoplasmic and membrane labeling; and 3 = membrane labeling)[12]. For p53, two different patterns were considered as aberrant expression: p53 overexpression, in which a strong nuclear staining was observed in at least 70% of tumor cells; and tumors with complete loss of p53 expression that include tumors with no staining or less than 5% staining. Stromal cells and benign epithelium served as control[13]. EBV status was determined by ISH, with probes against Epstein-Barr encoded RNA 1 (EBER1-Y5200). Tumor cell nuclei with dark-blue staining were considered positive.

All analyses were performed by two pathologists. If there was difference between these two observers, these slides were re-evaluated by both investigators.

Statistical analysis

Categorical and continuous variables were compared using the Chi-square or Fisher Exact Tests and ANOVA, respectively. Survival curves were estimated according to the Kaplan-Meier method and compared using the log-rank test. Alive patients were censored at the date of last contact. Postoperative mortality (defined as 30-d mortality in post-operative or during hospitalization) was omitted from the DFS analysis. Cox regression analyses, including hazard ratios with 95% confidence intervals, were performed to evaluate the prognostic association of clinicopathological characteristics with survival. P value < 0.05 was considered statistically significant. SPSS software (version 20.0) was used for statistical analysis.

RESULTS

A total of 287 patients were enrolled in the study, consisting of 168 (58.5%) men and 119 (41.5%) women. The mean age was 61.5 years. Most of the tumors had a distal location and subtotal gastrectomy was performed in 60.6% of cases. The most common macroscopic configuration was type III (48.4%) and intestinal *Laurén* (47.4%) the predominant histological type. The mean number of LN retrieved was 41.8, and LN metastases were presented in 56.4% of cases. Stage III was the predominant final stage, which occurred in 43.6% of the cases. Surgical mortality was 4.2%, neoadjuvant therapy was performed in 11.8% of cases, and 55.1% of patients received adjuvant therapy (Supplementary Table 1).

After IHC and ISH analysis for the 4 profiles evaluated (EBV, MSI, p53, E-cadherin), abnormal expression for a single profile was observed in 175 cases (60.9%), and 23 (8.1%) had alteration in two or more profiles. The remaining 89 cases (31%) showed no alteration in the assessed expression profile. Aberrant expression of p53 was observed in 105 cases (36.6%) (negative expression in 33 cases and strong expression in 92 cases), MSI in 60 cases (20.9%), and reduced or abnormal E-cadherin expression in 26 cases (9.1%). EBV positivity was present in 30 cases (10.5%). The protein expression and ISH results are summarized in Figure 1 and the microscopic findings in Figure 2.

The flowchart with the distribution of the subtypes according to TCGA and ACRG classifications are shown in Figure 3. According to TCGA classification, GCs were classified in 4 subtypes as follows: 30 (10.5%) EBV-positive; 58 (20.2%) MSI, 179

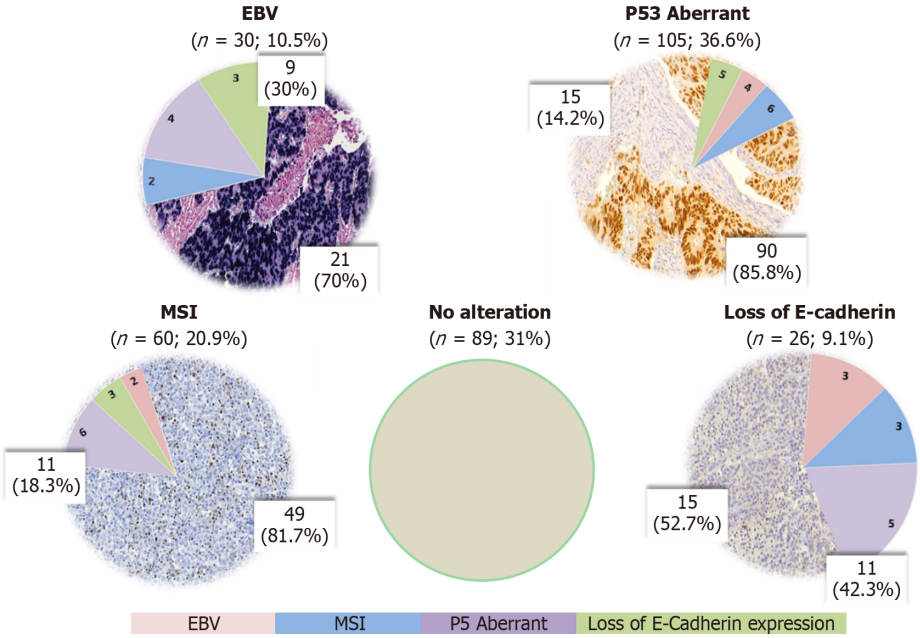


Figure 1 Results for Epstein-Barr virus infection by in situ hybridization, and for microsatellite instability, e-cadherin and p53 expression by immunohistochemistry. EBV: Epstein-Barr virus; MSI: Microsatellite instability.

(62.2%) CIN; and 20 (7%) GS.

Clinicopathological characteristics of GC according to the TCGA subtypes are demonstrated in Table 1. Proximal location ($P < 0.001$), total gastrectomy ($P = 0.001$) and increased inflammatory infiltrate ($P < 0.001$) were related to EBV subtype. MSI subtype was predominantly associated to advanced age ($P = 0.017$) and higher Charlson comorbidity index (CCI) ($P = 0.011$). While poorly differentiated histology ($P < 0.001$), *Laurén* diffuse type ($P < 0.001$), and advanced stage ($P = 0.029$) were characteristics related to GS subtype. Clinicopathological characteristics of GC based on ACRG classification subtypes are demonstrated in Supplementary Table 2. Unlike TCGA, ACRG subtypes were also significantly different regarding category pN (pathological lymph node metastasis), lymphatic, and venous invasion. Tumor size and inflammatory infiltrate were characteristics significant only in the TCGA.

Survival analysis

In a mean follow-up period of 37.9 mo, there were 79 recurrences and 95 deaths. GS and MSS/EMT subtype showed worse DFS and OS than the other subtypes. Conversely, MSI subtype had better survival in both classifications (Figure 4).

In multivariate analysis, type of gastrectomy, category pT, and the TCGA Classification subtypes were independent factors associated to DFS and OS (Table 2). Category pN were associated only to DFS. Regarding ACRG classification, using the EMT subtype as reference, only the MSI/p53 normal had a significant better DFS and OS (Supplementary Table 3).

DISCUSSION

The results of this study demonstrated that IHC and ISH techniques represent a feasible strategy to reproduce the GC molecular classification and can be applied in the daily diagnostic routine. We show that through the evaluation of the four main profiles (EVB, MSI, E-cadherin and p53), it was possible to define subtypes of tumors with distinct clinicopathological characteristics, similar to the findings of previous molecular classifications. Furthermore, we demonstrate the relationship between both TCGA and ACRG subtypes with long term survival submitted to curative D2 resection.

Considering the subtypes determined by both classification systems, EBV associated tumors represent the major point of disagreement between TCGA and ACRG. In the TCGA Classification, it is the first group to be discriminated[5,7]. Instead, ACRG Classification does not contemplate EBV GC as a subtype separately[6]. Importantly,

Table 1 Clinicopathological characteristics of gastric cancer patients according to the Cancer Genome Atlas classification

Variables	EBV	MSI	CIN	GS	*P* value
	n = 30 (10.5%)	*n* = 58 (20.2%)	*n* = 179 (62.2%)	*n* = 20 (7%)	
Age (yr)					0.017
mean (± SD)	61.4 (11.2)	65.9 (12.5)	60.1 (11.7)	60.6 (12.8)	
Sex					0.101
Male	23 (76.7)	29 (50)	103 (57.5)	13 (65)	
Female	7 (23.3)	29 (50)	76 (42.5)	7 (35)	
ASA Classification					0.753
I/II	27 (90)	50 (85.2)	160 (89.4)	19 (95)	
III/IV	3 (10)	8 (13.8)	19 (10.6)	1 (5)	
Charlson–Deyo comorbidity index					0.011
< 5	19 (63.3)	23 (39.7)	112 (62.6)	14 (70)	
≥ 5	11 (36.7)	35 (60.3)	67 (37.4)	6 (30)	
Tumor site					0.004
Upper	8 (26.7)	6 (10.3)	18 (10.1)	2 (10)	
Middle	7 (23.3)	8 (13.8)	34 (19)	3 (15)	
Lower	11 (36.7)	44 (75.9)	123 (68.7)	13 (65)	
Entire	4 (13.3)	0 (0)	4 (2.2)	2 (10)	
Type of resection					< 0.001
Subtotal	8 (26.7)	45 (77.6)	110 (61.5)	11 (55)	
Total	22 (73.3)	12 (22.4)	69 (38.5)	9 (45)	
Macroscopic type					0.244
I /II	9 (34.6)	19 (34.5)	37 (22.7)	4 (21.1)	
III/IV	17 (65.4)	36 (65.5)	126 (77.3)	15 (78.9)	
Tumor size (cm)					0.029
mean (± SD)	6.3 (4.4)	5.3 (2.9)	4.5 (3.0)	5.2 (2.9)	
Histological differentiation					0.001
Well/moderately	8 (26.7)	28 (48.3)	89 (49.7)	2 (10)	
Poorly differentiated	22 (73.3)	30 (51.7)	90 (50.3)	18 (90)	
Laurén type					< 0.001
Intestinal	11 (36.7)	34 (58.6)	88 (49.2)	3 (15)	
Diffuse/mixed	12 (40)	22 (37.9)	88 (49.2)	16 (80)	
Undetermined	7 (23.3)	2 (3.4)	3 (1.7)	1 (5)	
Tumor invasion					0.277
pT1/pT2	10 (33.3)	29 (50)	70 (39.1)	6 (30)	
pT3/pT4	20 (66.7)	29 (50)	109 (60.9)	15 (70)	
pN status					0.147
pN negative	14 (46.7)	32 (55.2)	73 (40.8)	6 (30)	
pN positive	16 (53.3)	26 (44.8)	106 (59.2)	14 (70)	
Lymphatic invasion					0.156
Absent	16 (53.3)	22 (37.9)	96 (53.6)	8 (40)	
Present	14 (46.7)	36 (62.1)	83 (46.4)	12 (60)	

Venous Invasion					0.300
Absent	22 (73.3)	35 (60.3)	125 (69.7)	11 (55)	
Present	8 (26.7)	23 (39.7)	54 (30.3)	9 (45)	
Perineural invasion					0.267
Absent	14 (46.7)	36 (62.1)	91 (50.8)	8 (40)	
Present	16 (53.3)	22 (37.9)	88 (49.2)	12 (60)	
Peritumoral inflammatory infiltrate					< 0.001
Absent/mild	8 (26.7)	35 (60.3)	131 (73.2)	17 (85)	
moderate/intense	22 (73.3)	23 (39.7)	48 (26.8)	3 (15)	
pTNM stage					0.014
I/II	18 (60)	40 (69)	93 (52)	6 (30)	
III/IV	12 (40)	18 (31)	86 (48)	14 (70)	

ASA: American Society of Anesthesiologists; CIN: Chromosomal instability; EBV: Epstein-Barr virus; GS: Genomically stable; MSI: Microsatellite instability; SD: Standard deviation.

EBV positive GC has been frequently associated to high programmed death-ligand 1 (PD-L1) expression. Since this subtype is a potential candidate for immunotherapy[10,14], it becomes clinically interesting to maintain EBV positive tumors as a distinct subtype. Proximal location, larger diameter lesions, and predominance of *Laurén*'s undetermined and poorly differentiated tumors are also features commonly associated with positive EBV tumors, and also found in our study. We also observed that there was a significantly higher frequency of total gastrectomy in this subtype, reinforcing its proximal predilection and larger dimensions. In relation to morphological aspects, the EBV subtype exhibited a predominance of intense inflammatory infiltrate, defined as gastric "lymphoepithelioma-like" carcinoma, which have a solid pattern and are classified as poorly differentiated[15].

Conversely, MSI status represents a profile considered in both classifications. MMR proteins assessment by immunoexpression is a well-established and efficient technique to determine MSI profile. It is commonly used in pathological routine evaluation for colorectal tumors, since The American College of Gastroenterology recommends screening for DNA dMMR in all newly diagnosed cases of colorectal cancer[16]. Compared to polymerase chain reaction-based analysis, IHC demonstrated sensitivity of 91.1% and specificity of 98.5% in the detection of MSI phenotype in GC [17].

GCs with MSI have been associated to some particular characteristic, including advanced age, intestinal histological type, location in the middle and distal third, and less advanced stages[18]. Similar to literature, we found that patients were older and consequently presented more comorbidity according to CCI. Tumors in our cohort were more frequently localized in the distal third of the stomach and more likely to undergo subtotal resections, with predominance of the *Laurén* intestinal type. MSI GC was the subtype that presented better survival rates. The reason why tumors associated to MSI usually have a better prognosis is not yet fully elucidated. It is believed that in these patients the lymphocytic peritumoral infiltrate, in response to peptides generated by MSI tumors, plays an important role in the anti-tumor response by inducing tumor cell apoptosis through cytokine stimulation[19]. The best prognosis can also be attributed to some characteristics related to these tumors, as the lower LN involvement, which is a hallmark of this subtype. Even in cases with pN+ status, metastases usually affect a smaller number of LNs and stations, besides being located closer to the primary tumor[20].

An additional, noteworthy aspect is related to the efficacy of fluopyrimidine/platin-based CMT in locally advanced GC with MSI. The CMT in these patients did not provide survival benefit[16,21]. Also, MSI GC patients who received perioperative CMT were associated with worse OS, further suggesting a deleterious effect with the addition of treatment in this subgroup of tumors. On the other hand, the immuno-therapy for MSI-associated tumors has been recently approved, regardless of tumor site, and has shown a positive impact on the survival of these patients. Thus, the availability of the evaluation in the current diagnostic practice, as well as its impact on the CMT regime, makes the identification of the MSI subtype the main candidate for

Table 2 Univariate and multivariate analysis for disease-free survival and overall survival-including the Cancer Genome Atlas subgroups

Disease-free survival	Univariate			Multivariate		
Variables	**HR**	**95%CI**	***P* value**	**HR**	**95%CI**	***P* value**
Male (*vs* female)	0.93	0.59-1.46	0.745	–	–	–
Age > 65 (*vs* < 65 yr)	1.52	0.95-2.45	0.082	–	–	–
Charlson < 5 (*vs* ≥ 5)	0.65	0.40-1.05	0.076	–	–	–
Total Gastrectomy (*vs* subtotal)	2.33	1.49-3.62	< 0.001	1.8	1.12-2.88	0.015
Diffuse/mixed (*vs* others)	1.80	1.15-2.83	0.011	1.24	0.78-1.97	0.367
pT3/pT4 status (*vs* pT1/pT2)	10.87	4.72-24.98	< 0.001	5.64	2.33-13.64	< 0.001
pN+ (*vs* pN0)	7.56	3.77-15.15	< 0.001	3.25	1.54-6.80	0.002
CMT (*vs* none)	1.28	0.82-1.98	0.280	–	–	–
TCGA Classification						
GS	1	–	–	–	–	–
EBV	0.42	0.17-1.06	0.068	0.37	0.14-0.94	0.037
MSI	0.21	0.09-0.52	0.001	0.31	0.13-0.76	0.010
CIN	0.43	0.21-0.84	0.014	0.43	0.22-0.87	0.018
Overall survival	Univariate			Multivariate		
Variables	HR	95%CI	*P* value	HR	95%CI	*P* value
Male (*vs* female)	1.08	0.72-1.63	0.708	–	–	–
Age > 65 (*vs* < 65 yr)	1.16	0.77-1.74	0.483	–	–	–
Charlson < 5 (*vs* ≥ 5)	1.16	0.84-1.88	0.273	–	–	–
Total Gastrectomy (*vs* subtotal)	1.90	1.27-2.84	0.002	1.7	1.11-2.60	0.014
Diffuse/mixed (*vs* others)	1.56	1.04-2.34	0.032	1.16	0.77-1.77	0.765
pT3/pT4 status (*vs* pT1/pT2)	4.50	2.59-7.82	< 0.001	3.23	1.74-5.97	< 0.001
pN+ (*vs* pN0)	3.29	2.02-5.35	< 0.001	1.69	0.98-2.92	0.061
CMT (*vs* none)	0.96	0.64-1.44	0.841	–	–	–
TCGA Classification						
GS	1	–	–	–	–	–
EBV	0.33	0.13-0.82	0.017	0.27	0.10-0.68	0.006
MSI	0.27	0.12-0.61	0.001	0.35	0.15-0.78	0.01
CIN	0.46	0.24-0.88	0.019	0.47	0.25-0.91	0.025

CI: Confidence interval; CIN: Chromosomal instability; CMT: Chemotherapy; EBV: Epstein-Barr virus; GS: Genomically stable; HR: Hazard ratio; MSI: Microsatellite instability.

incorporation into the routine evaluation.

On the other hand, define the aberrant expression of p53 by IHC is a greater challenge. Different criteria have already been employed[22]. Nuclear localization is essential for p53 activity, and its nuclear accumulation may result from increased regulation of wild-type expression or decreased degradation in response to cellular stresses, including DNA damage. The presence of wild-type protein expression is a normal physiological response to decrease the G1 cell cycle and allow the repair of damaged DNA. Therefore, low levels of the wild-type p53 protein can be detected in the nucleus by IHC. In contrast, mutations in TP53 result in the production of proteins with a prolonged half-life that accumulates in the nucleus, causing its overexpression. Conversely, some missense or point mutations can result in a truncated protein that is not stable enough to cause any detectable nuclear accumulation. Thus, the aberrant expression of p53 can be defined either by the absence or exacerbated nuclear expre-

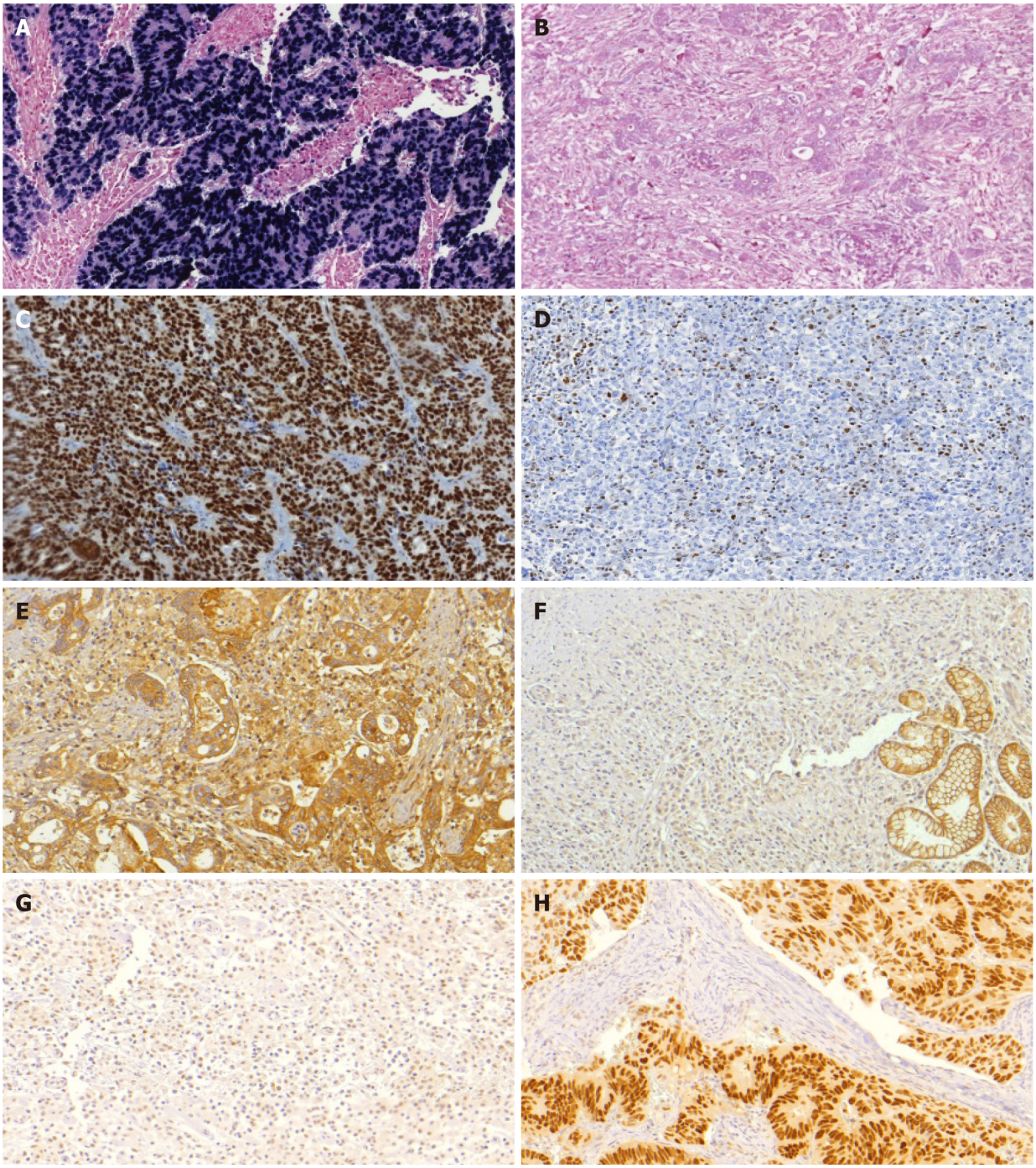


Figure 2 Microscopic findings in gastric cancer cases. A: Tumor positive for Epstein-Barr virus (EBV) by *in situ* hybridization; B: Gastric cancer (GC) negative for EBV infection; C: Imuno-histoquímico (IHC) analysis of MLH1 expression in tumors with retained expression of MLH1; D: Loss of MLH1 expression; E: GC with preserved E-cadherin expression; F: tumor exhibiting loss of E-cadherin expression; G: Normal IHC expression of p53; H: tumor with aberrant p53 expression.

ssion[13]. As a characteristic, tumors with this phenotype are generally associated with intestinal histology, which was also demonstrated in our results when comparing the MSS/p53 and MSS/p53- groups from ACRG classification.

Finally, the subtype of GC associated with E-cadherin aberrant expression showed poorly differentiated histology, predominance of diffuse tumors, LN metastasis at more advanced stages, and worse survival[6,9]. E-cadherin is an essential adhesion molecule for the consolidation of epithelial architecture, maintenance of cell polarity, and differentiation during fetal development and in adult life. It acts as a broad-acting tumor suppressor. It is suggested that loss of E-cadherin expression is the phenotypic expression of CDH1 and RHOA mutations[23]. IHC expression of E-cadherin is evaluated by the presence of continuous and linear membrane staining at the boundaries between cells, as well as at the base of gastric gland. The altered expression can

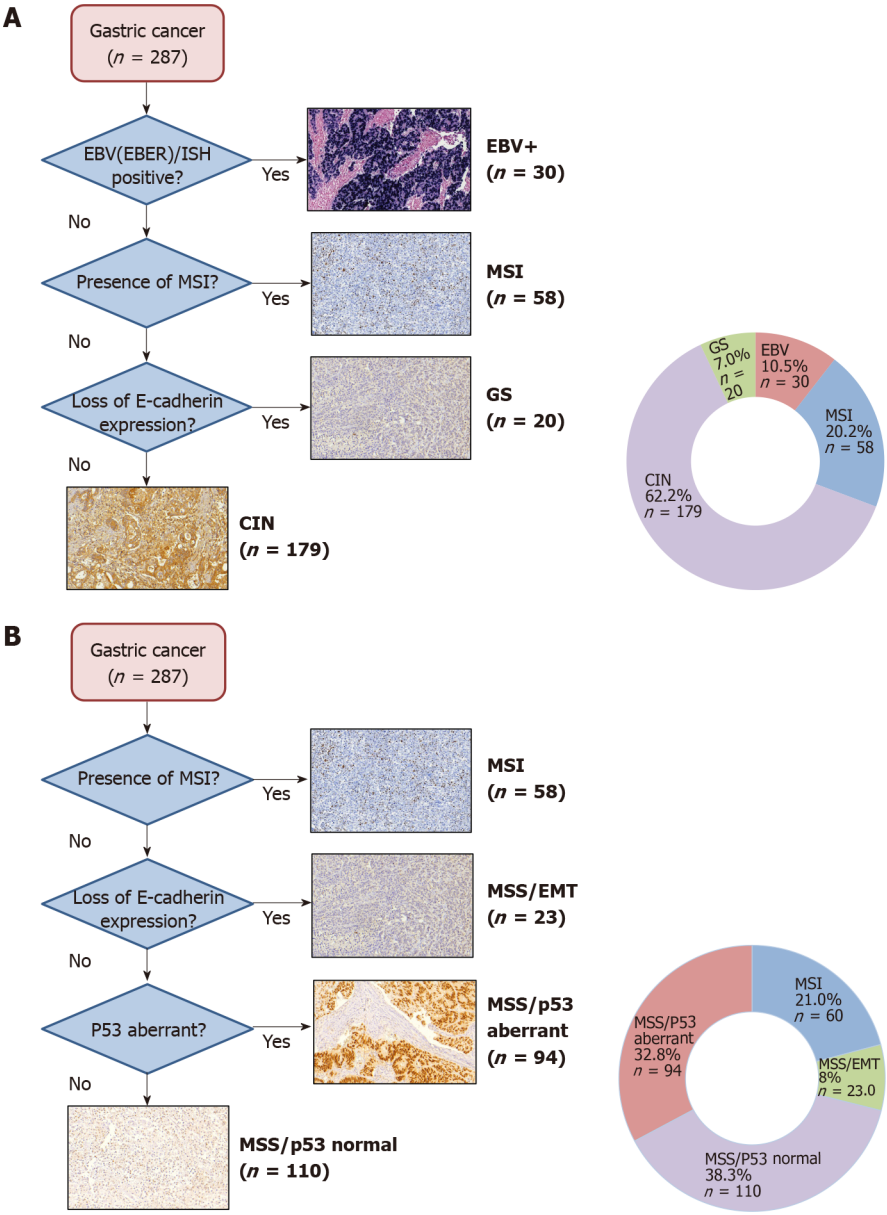


Figure 3 Flowchart showing the classification of molecular subtypes and final distribution. A: Classification by TGCG; B: Asian Cancer Research Group classification. EBV: Epstein-Barr virus; EMT: Epithelial to mesenchymal transition; GS: Genomically stable; ISH: In situ hybridization; MSI: Microsatellite instability; MSS: Microsatellite stable.

be evidenced by the total or partial loss of cell membrane expression or by aberrant cytoplasmic expression[12]. As in the analysis of p53, E-cadherin evaluation is subjective, but presents better homogeneity among different studies[24].

It is important to emphasize that the association of E-cadherin expression and alterations in the *CDH1* gene is not yet clearly defined. Only a minority of GC cases actually have a mutation in the CDH1 gene. Somatic mutation has been reported in about 3% to 50% of GC with diffuse histology[25]. It is estimated that 70% of patients with absence of E-cadherin expression do not have a CDH1 mutation. This fact suggests that the E-cadherin expression may be influenced by the interaction of other post-translational mechanisms[26]. In patients with hereditary diffuse GC (HDGC) who already have a germline mutated CDH1 allele, the hypermethylation of the CDH1 allele is the most common second-hit cause for complete inactivation of the gene[27].

In summary, the analysis through IHC and ISH techniques has proven to be feasible, but is also subject to technical limitations. It involves the use of different antibodies and probes, tissue preparation, and evaluation criteria. In addition, TMA technique allows only the representation of some areas of the tumor, which may lead to a variation in results considering the tumor heterogeneity[28]. One of the main aspects that must be considered is that the same tumor may present alterations that

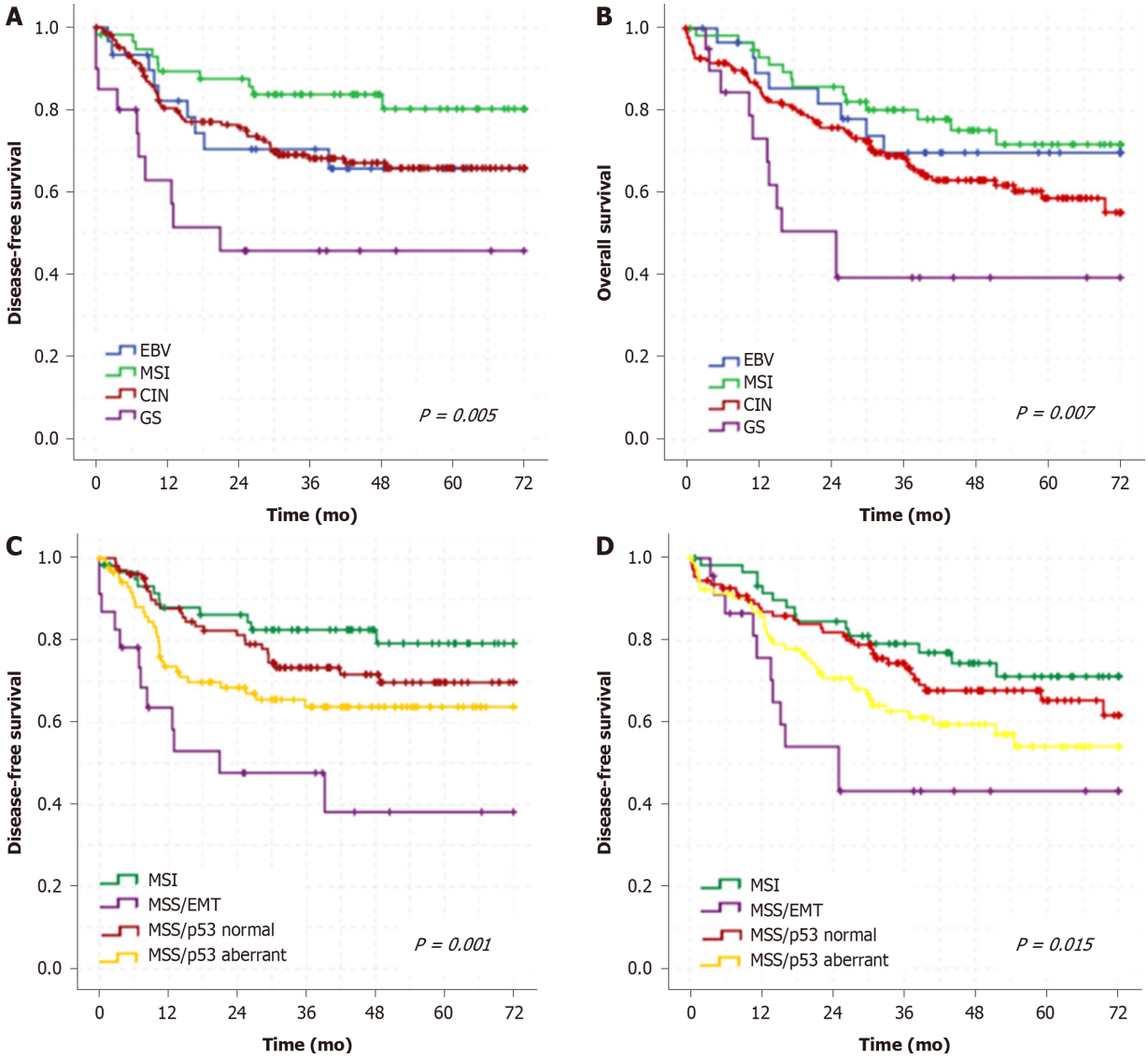


Figure 4 Disease-free survival and overall survival according to the Cancer Genome Atlas and Asian Cancer Research Group classification. A: Disease-free survival according to the Cancer Genome Atlas (TCGA) classification; B: Overall survival according to TCGA classification; C: Disease-free survival according to Asian Cancer Research Group (ACRG) classification; D: Overall survival according to ACRG classification. CIN: Chromosomal instability; EBV: Epstein-Barr virus; EMT: Epithelial to mesenchymal transition; GS: Genomically stable; MSI: Microsatellite instability; MSS: Microsatellite stable.

correspond to two or more profiles[21,29]. Thus, the definition of which subtype belongs to the patient with more than one profile will depend on the chosen molecular classification flowchart.

As we only analyze the four main profiles related to the subtypes of GC, some cases may be misclassified, since GCs may have other alteration in markers not evaluated, such as RHOA, related to GS subtype, and tyrosine kinase receptor, related to CIN subtype. Also, limitations in relation to the technique and immunohistochemical markers may lead to the misclassification some GC in the subtypes, particularly in terms of the heterogeneous staining pattern. Taking into account the importance and the usefulness of IHC markers, validating those already identified is necessary to integrate surgical and molecular pathology with clinical medicine. Another limitation of the classification based on protein expression is the impossibility to determine if the change in expression pattern is due to epigenetic factors (such as gene silencing, frequent in MLH1, for example) or genetic factors. Furthermore, we cannot define if a genetic alteration is somatic or germinative, an important issue in the context of tumors with aberrant E-cadherin, which may be related to HDGC.

As strengths, in the present study we used data collected prospectively from a database research project, minimizing the possibility of classification bias of the variables. In order to make the studied population more homogeneous in relation to surgical treatment and to reduce selection bias, all consecutive patients submitted to

resection with curative intent and D2 Lymphadenectomy were included in the selected period, which provided a better prognostic prediction. Accordingly, the results obtained in this study add evidence to the literature that the effectiveness of both ISH and IHC staining to define GC subtypes will allow the adoption of a more individualized therapeutic approach[30]. The ToGA trial represented a major milestone in this regard, after demonstrating improved survival of GC HER2-positive treated with Trastuzumab with palliative intent[31]. Recently, Pembrolizumab was approved by the FDA for patients with unresectable, metastatic, or locally recurrent GC with MSI profile and/or PD-L1 positive[32-34]. Certainly, new targeted therapy drugs for specific tumoral markers and immune checkpoints will emerge in the near future, keeping GC molecular classifications as a major issue of interest and investigation.

CONCLUSION

The IHC/ISH analysis was able to distinguish immunophenotypic groups of GC with distinct clinicopathological characteristics and prognosis, resembling the subtypes of the molecular classifications. TCGA subtypes had different outcomes, where MSI subtype was associated with a better prognosis and GS had worse survival outcomes. Accordingly, this method of classification may allow an easier definition of GC subtypes in daily clinical practice, contributing to the therapeutic planning, driving target therapy and individualized treatment.

ARTICLE HIGHLIGHTS

Research background

Molecular classification of gastric cancer (GC) emerged as a promising option to define therapeutic strategies and prognostic subgroups. However, the costs and technical complexity of molecular methodologies remains an obstacle to its adoption. Thus, their clinical significance by other approaches needs further evidence, especially in the western GC.

Research motivation

Although molecular classifications as proposed by The Cancer Genome Atlas (TCGA) Research Network Group and Asian Cancer Research Group (ACRG) subtypes provided a guide for patient stratification and trials of targeted therapy, the clinical utility of these classifications is still limited due to the technical complexity, as indicated by the fact there is little evidence of its application in a real-world setting.

Research objectives

The purpose of this study was to determine the subgroups of molecular classification by immunohistochemistry (IHC) and in situ hybridization (ISH), and to evaluate its characteristics and impact on long-term survival in a Western cohort of GC patients treated with curative intent.

Research methods

We retrospectively evaluated GC patients who underwent D2-gastrectomy between 2009 and 2016 by tissue microarray. Cases were assessed for microsatellite instability (MSI) status, E-cadherin and p53 expression by IHC and for Epstein-Barr virus (EBV) by ISH. Clinicopathological characteristics and survival of GC were evaluated according to TCGA and ACRG classification system.

Research results

A total of 287 GC patients were included. Based on IHC and ISH analysis, 5 profiles were defined as follows: E-cadherin aberrant (9.1%), MSI (20.9%), p53 aberrant (36.6%), EBV positivity (10.5%), and p53 normal (31%), which corresponded to tumors that showed no alteration in other profiles. Flowchart according to the TCGA and ACRG classifications were used to define the subtypes, where clinical and pathological characteristics associated with GC subtypes were evidenced. In both classifications, the MSI group had better survival. While the subtype represented by the loss of e-cadherin expression (subtype GS and microsatellite stable/epithelial to mesenchymal transition) was related to a worse prognosis. The classification proposed by the TCGA

was an independent factor associated with survival in patients with GC.

Research conclusions

The IHC/ISH analysis was able to distinguish immunophenotypic groups of GC with distinct characteristics and prognosis, and is a viable option for use in a clinical setting.

Research perspectives

The identification of gastric adenocarcinoma subtypes through techniques available in clinical practice and the development of integrated approaches for diagnostic applications, such as prognosis prediction or response to therapy intervening, emerge as an essential phase toward personalized medicine in GC treatment.

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CASE REPORT

PIK3CA mutation in non-metastatic triple-negative breast cancer as a potential biomarker of early relapse: A case report

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Abstract

BACKGROUND

Currently, the detection of PIK3CA mutations is of special interest in personalized medicine because it is frequently found in triple-negative breast cancer (TNBC). The PI3KCA mutation is an independent negative prognostic factor for survival in metastatic breast cancer, and its prognostic value in liquid biopsy as a biomarker of treatment and early relapse is under investigation, both for metastatic disease and neoadjuvant scenario with curative intent.

CASE SUMMARY

A 54-year-old female patient with TNBC clinical stage IIIA, who, after receiving neoadjuvant chemotherapy (based on anthracyclines and taxanes), surgery, radiotherapy, and adjuvant capecitabine, was detected with a PI3KCA mutation in tissue and peripheral blood (ctDNA in liquid biopsy). After 10 mo, the patient had disease relapse of left cervical node disease.

CONCLUSION

The detection of PIK3CA mutation in TNBC after neoadjuvant treatment might be associated with early relapse or rapid disease progression.

Key Words: Triple-negative breast cancer; PIK3CA mutation; Liquid biopsy; Early relapse;

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Core Tip: This case report evaluates the detection of a PIK3CA mutation in liquid biopsy and tumor tissue in a patient with locally-advanced triple-negative breast cancer after receiving conventional oncological therapy and its association with early relapse and progression disease. This case highlights the importance of detecting the PIK3CA mutation as a potential biomarker of early relapse. In addition, the PIK3CA mutation could lead to additional interventions to detect metastatic disease earlier in the follow-up. There is limited information of liquid biopsy studies after surgery. Furthermore, there is very limited data about trials of PIK3CA mutations in Peruvian patients with non-metastatic breast cancer.

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INTRODUCTION

Triple-negative breast cancer (TNBC) is a highly heterogeneous disease, with limited options of medical treatment. Currently, there is a great interest in the need to investigate the presence of predictive and/or prognostic biomarkers, as well as new personalized therapies. Despite the initial sensitivity to neoadjuvant chemotherapy, TNBC patients are more likely than other subtypes to develop relapse within the first 3 years after diagnosis, both distantly and locally, usually with visceral and brain metastases[1].

The phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway is activated in some cancers (including breast cancer) and is important not only as a biomarker, but also as a possible therapeutic target in TNBC. Large-scale genomic analyses have characterized the heterogeneous nature of this subtype, identifying a subgroup with activation of the PI3K/AKT pathway through somatic mutations in the PIK3CA gene, which induces tumorigenesis and angiogenesis[2]. PIK3CA mutations are found in a 28%-40% of patients with breast cancer, while in the luminal androgen receptor TNBC subtype (associated with high mutational load), it presents mutations enriched in PIK3CA up to a 55%[3-5]. The complexity of the PI3K pathway, as well as the presence of multiple mediators within the same signaling pathway, explain part of the resistance to treatment, as well as the risk of disease relapse. The PIK3CA mutation is considered an independent negative prognostic factor in advanced breast cancer. It can be detected using tumor tissue or liquid biopsy[6].

CASE PRESENTATION

Chief complaints

Fifty-four-year-old female patient, postmenopausal, with a family history of a maternal aunt with breast cancer (62 years).

History of present illness

Patient detected a tumor in her left breast of 3 years of evolution, without pain, without nipple involvement nor changes in color.

History of past illness

The patient has controlled hypertension and diabetes mellitus.

Physical examination

Clinical examination showed an Eastern Cooperative Oncology Group (ECOG) performance status of 1. Lymphatics: 6 cm left axillary conglomerate. Breasts: Two tumors in the left breast, one in R12 of 5.5 cm, at 2 cm from the nipple, the other in R2 of 2.5 cm, without skin involvement.

Imaging examinations

The mammography and breast ultrasound showed signs suggestive of left breast multicentric compromise associated with ipsilateral nodal involvement. The Breast Imaging-Reporting and Data System (BIRADS) score was 6.

Chest computer tomography (CT) showed signs suggestive of a malignant neoformative process of the left breast associated with ipsilateral nodal involvement (Figure 1). Abdominal CT and bone scans were without metastatic lesions.

Pathology

The core biopsy of the left breast reported the following: The R12 lesion (Figure 2) corresponds to an infiltrating carcinoma of the breast, invasive ductal carcinoma not otherwise specified (NOS)/invasive breast carcinoma of no special type (NST) with lobular pattern infiltration areas, and histological grade noted high, lymphovascular invasion was present, but perineural invasion was not observed. Immunohistochemistry analysis determined that the tumor was negative for estrogen receptor (ER), progesterone receptor (PR), cerbB2 (or human epidermal growth factor, HER2) , positive for e-cadherin, and had 60% positive for ki67 (Figure 3). The lesion in R2 corresponds to an infiltrating carcinoma of the breast, NOS/NST with areas of infiltration of lobular pattern, and histological grade noted that neither high, lymphovascular infiltration or perineural invasion were observed. Immunochemistry analysis had similar characteristics to those described in radius 12 (R12; clockwise), with negative staining for ER, PR, cerbB2, positive for e-cadherin, and 60% positive for ki67 In both lesions, significant intertumoral lymphocytic population less than 10% was not identified.

FINAL DIAGNOSIS

The final diagnosis was TNBC IIIA with left breast multicentric compromise.

TREATMENT

The patient began standard neoadjuvant therapy (NAT) (doxorubicin-cyclophosphamide for 4 courses, followed by weekly paclitaxel for 12 wk) reaching partial clinical response (R12: tumor 4 cm × 2 cm, R2: 1.5 cm tumor, left axillary node: 2 cm × 2 cm).

The patient underwent a modified radical mastectomy with radical axillary dissection. The biopsy indicated that R12 was an infiltrating ductal carcinoma, NOS/NST, histological grade III, with lymphovascular infiltration. The larger diameter of the invasive component showed the following: 40 mm × 32 mm, pT2 (Figure 4), free section borders, immunohistochemistry in relation to TNBC. In R2, a TNBC of similar characteristics to the pathology of R12 was reported, with a larger diameter of the invasive component of 6 mm × 4 mm. Macrometastases and extracapsular extension of lymph nodes 12 and 16 were detected, pN3a (Figure 5). The residual cancer burden group after neoadjuvant therapy (NAT) was III. In addition, tumor-infiltrating lymphocytes (TILs) were performed on residual tissue (post-neoadjuvant scenario), with a count of less than 10% (Figure 6). CD8 Lymphocyte staining showed a count of 40% (Figure 7).

Subsequently, the patient received external radiotherapy at a dose of 5000 cGy in 25 sessions at the level of the left rib cage and ipsilateral supraclavicular region, and continued with adjuvant capecitabine, completing 6 mo of treatment. She has continued with follow-up since May 2018 after negative images for the disease.

After surgery, she was included in two biomarker local studies. The first research study, called "Evaluation of prognostic markers of survival and predictive of treatment response in cancer population through the first platform in Peru for the detection and gene determination without amplification of DNA or RNA", carried out an analysis in tumor tissue with nCounter® Pan Cancer from a panel that included 770 genes,

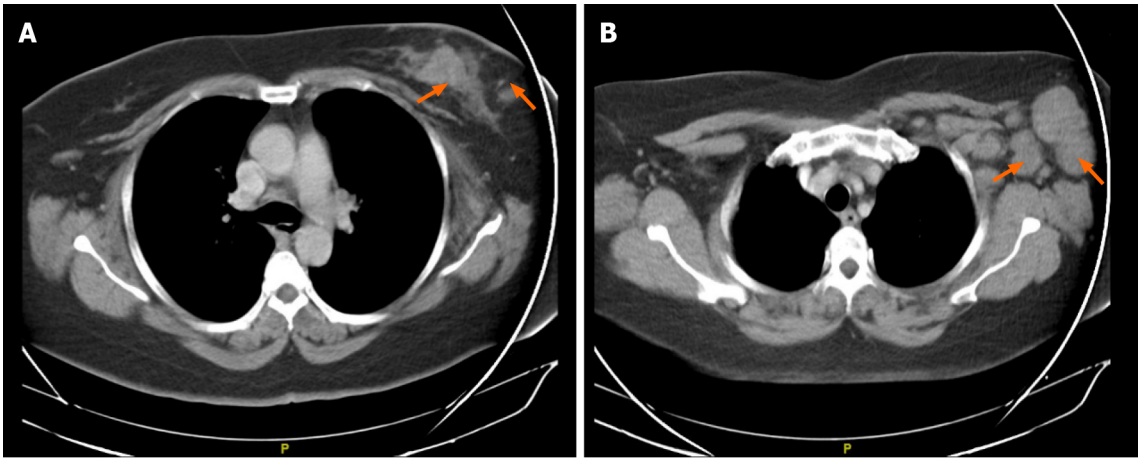


Figure 1 Chest computer tomography scan used as part of staging. A: Left breast with heterogeneous area on the left side suggestive of a 38 mm malignant neoformative neoplasm; B: Associated with ipsilateral nodal involvement.

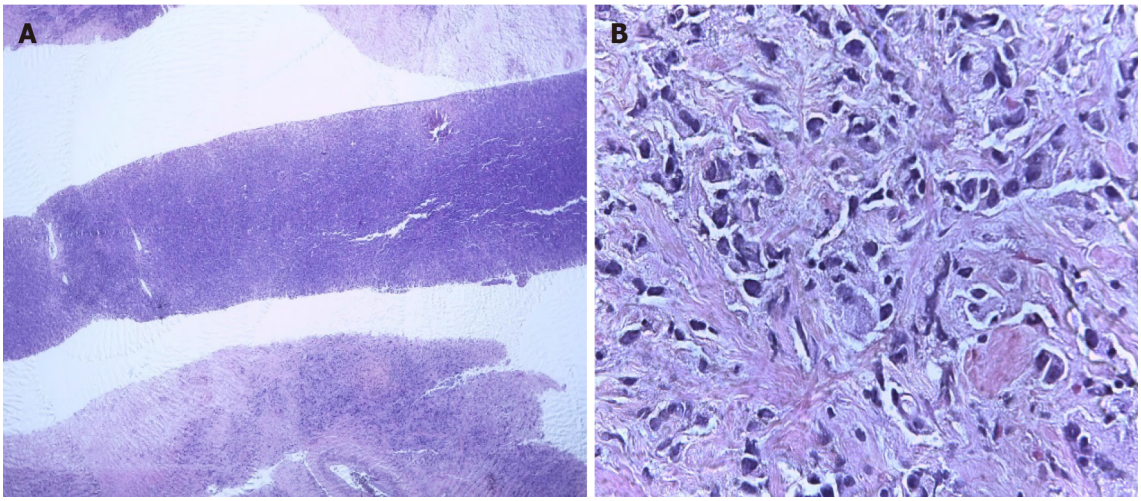


Figure 2 Core biopsy of the left breast. A: R12 tumor sample; B: Hematoxylin-eosin staining.

showed the presence of high "anergic" (exhausted) CD8 (Figure 8)[7].

The other study was conducted using a liquid biopsy and called: "Circulating tumor DNA as biomarkers in triple negative breast cancer", resulted in the detection of PIK3CA H1047R (110 alpha), which was mutated, both in liquid biopsy (Figure 9) and in paraffin tissue (Figure 10)[8].

OUTCOME AND FOLLOW-UP

In March 2019, after a 10-mo disease-free interval, the patient's disease relapsed with the presence of multiple lymphadenopathies in the left cervical region, the largest at 3 cm. Biopsy of the left cervical node indicated metastases of infiltrating primary breast carcinoma with an intermediate histological grade (Figure 11A), and immunohistochemistry analysis in relation to TNBC finding 60%-70% positive cells for ki67 (Figure 11B). However, chest and abdomen tomography without signs of visceral recurrence. Genetic counseling was requested, but the patient was negative for the BRCA mutation. The patient started chemotherapy with carboplatin-gemcitabine for 6 courses, reaching a complete clinical response and reassessment imaging without evidence of disease. She continued maintenance with gemcitabine.

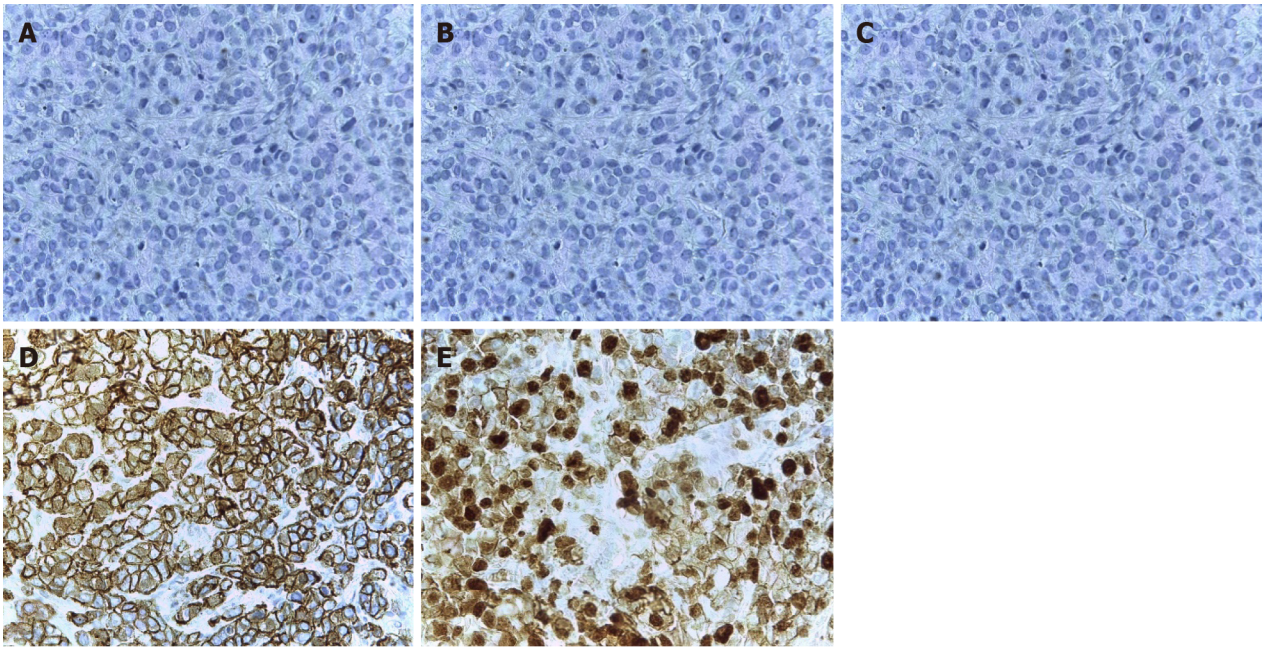


Figure 3 Immunohistochemistry of the R12 left breast. A: Estrogen receptor: Negative; B: Progesterone receptor: Negative; C: CerbB2: Negative; D: E-cadherin: Positive; E: Ki67: 60%.

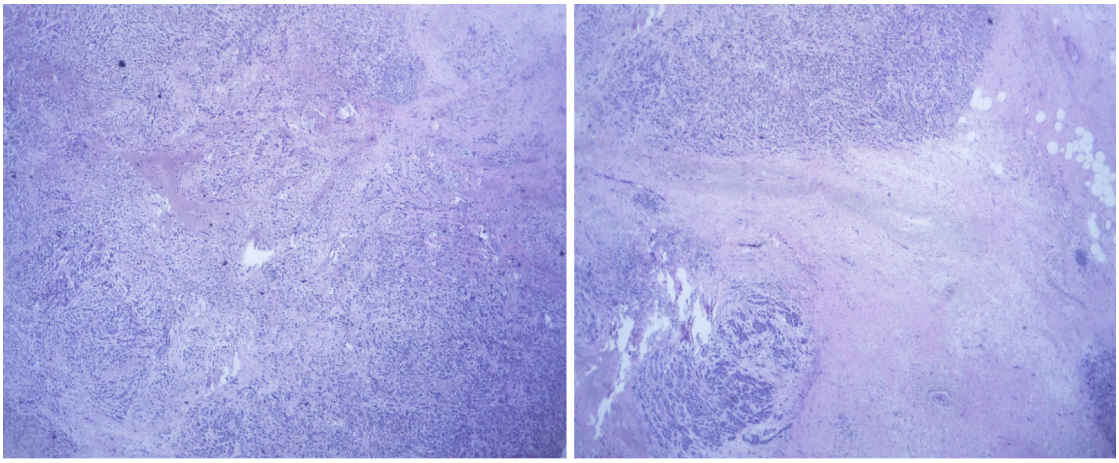


Figure 4 Surgical piece: R12 left breast tumor.

DISCUSSION

Patient with TNBC IIIA with left breast multicentric compromise, who, after receiving standard neoadjuvant treatment, followed by surgery, radiotherapy, and adjuvant capecitabine, presents cervical lymph node recurrence after a 10-mo disease-free interval. After surgery, she participated in 2 biomarker research studies, detecting the presence of a positive PIK3CA mutation in tumor tissue and peripheral blood (liquid biopsy).

The PI3K/AKT/mTOR pathway is an oncogenic intracellular pathway that regulates cell proliferation, metabolism, growth, survival, and apoptosis. The PIK3CA mutation, being the most common of the PI3K/AKT signaling pathway amplifications, is present in all molecular subtypes of breast cancer, and is detected in 20%-25% of all cases[2]. Although these individual mutations are rare, the combined activated mutations in PIK3CA and AKT1, with inactivating mutations in PTEN (a tumor suppressor gene that is inactivated when mutations are detected), occur in 25%-30% of metastatic TNBC (mTNBC)[2]. It has been determined that the PIK3CA mutation has an independent negative prognostic value of survival, as a systematic review has shown that patients with breast cancer and high levels of ctDNA after receiving neoadjuvant treatment have early disease relapse[9-11].

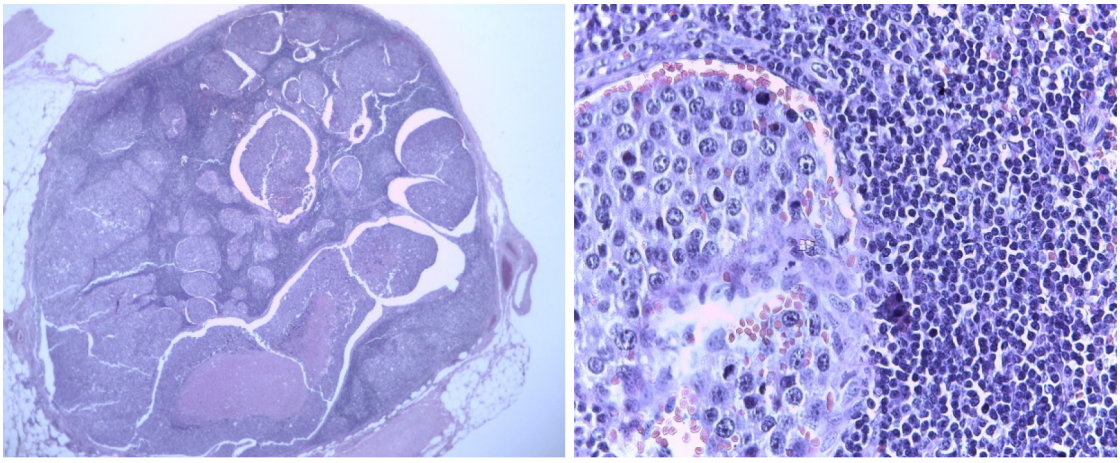


Figure 5 Surgical piece: Left axillary node.

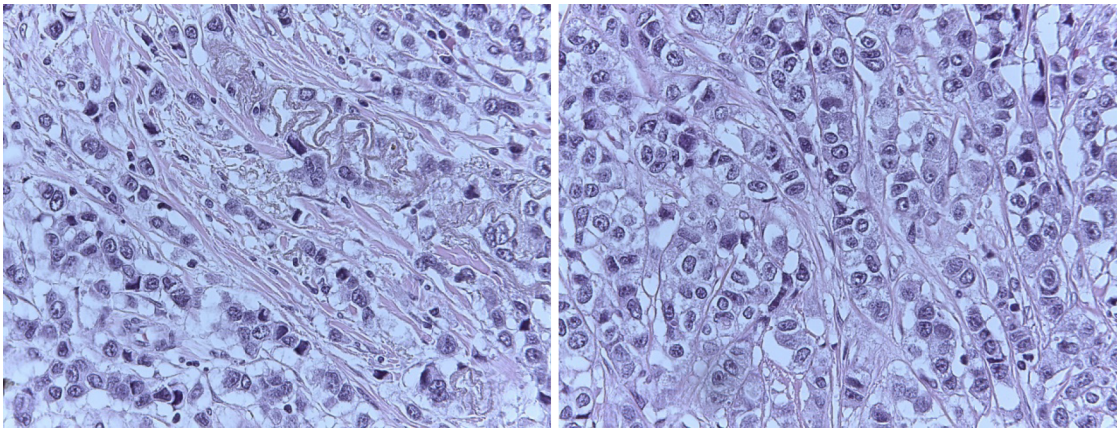


Figure 6 Tumor-infiltrating lymphocytes in residual tissue (post-neoadjuvant scenario): Less than 10%.

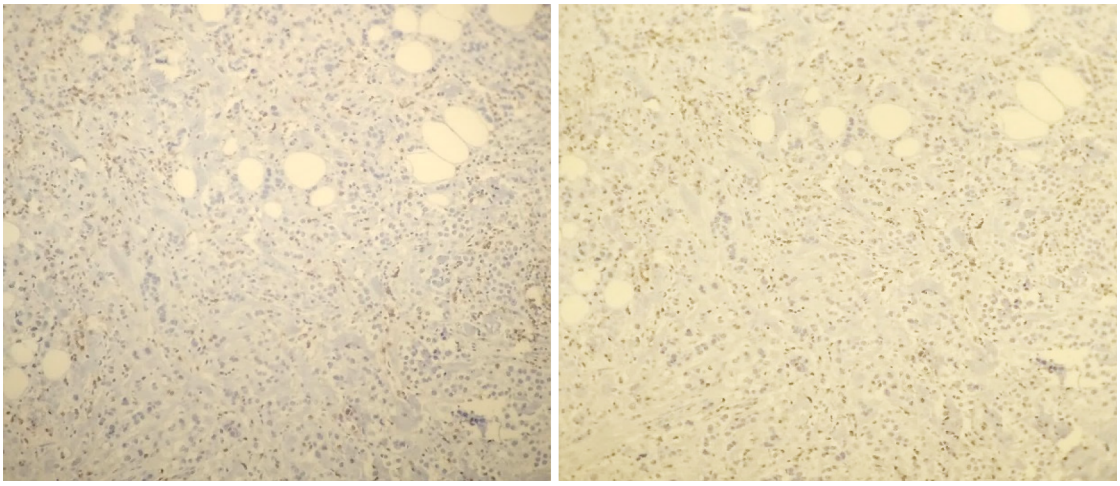


Figure 7 CD8 staining (× 10 magnification): 40%.

Liquid biopsy is being evaluated in breast cancer as a potential tool to capture tumor evolution in real time, to guide and monitor systemic treatment, as well as being a promising method to identify drug resistance mechanisms through the detection of genomic alterations[9,10]. ctDNA corresponds to a small fraction (0.01%-10%) of short cell-free DNA fragments from tumors and are detectable in body fluids (for example: Blood, saliva, or urine)[11]. In TNBC, there is evidence that evaluation of

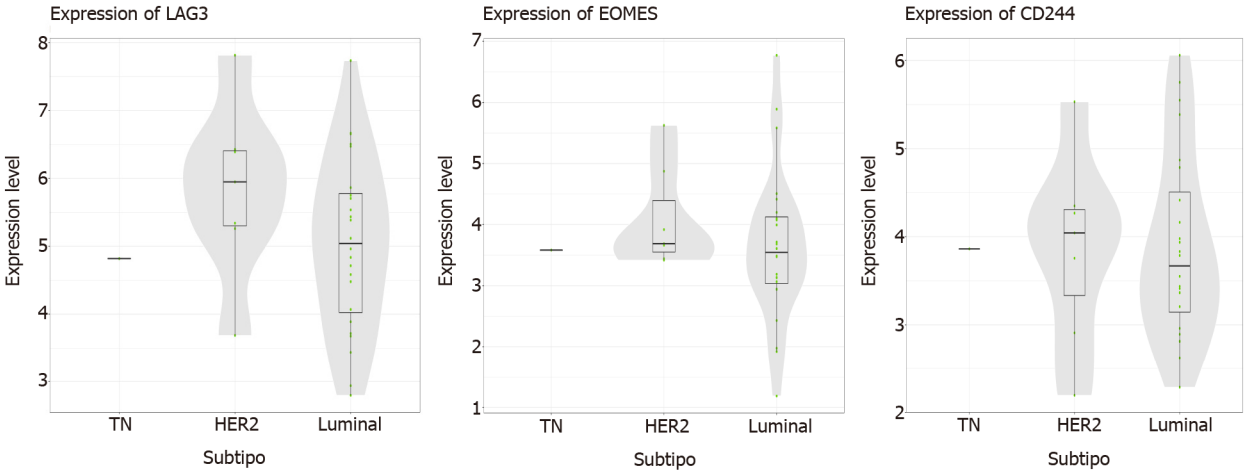


Figure 8 List of genes determining the presence of anergic CD8 cells ("exhausted"): "high" result, from the nCounter® PanCancer Immune Profiling Panel platform.

Liquid biopsy
PIK3CA E542K (110 alpha): no mutated
PIK3CA E545K (110 alpha): no mutated
PIK3CA H1047R (110 alpha): **mutated**

PI3KCA H1047R
(Puntos azules indican presencia de la mutación)
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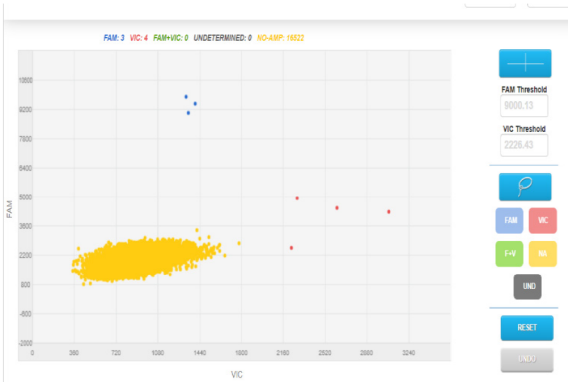


Figure 9 Liquid biopsy, PIK3CA 110 alpha result: Mutated.

Paraffined tissue
PIK3CA E542K (110 alpha): no mutated
PIK3CA E545K (110 alpha): no mutated
PIK3CA H1047R (110 alpha): mutated

PI3KCA H1047R (Curva de amplificación)
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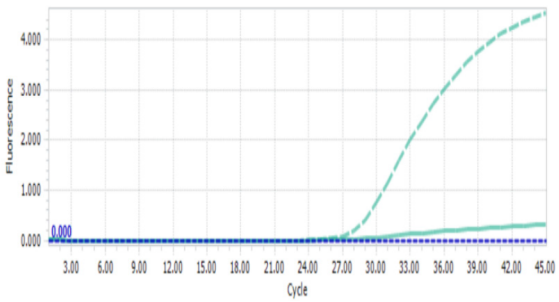


Figure 10 Paraffined tissue, PIK3CA 110 alpha result: Mutated.

mutated PIK3CA in circulating tumor DNA (ctDNA) using liquid biopsy has a potential prognostic value as a biomarker, including diagnosis and follow-up during treatment (response to NAT)[12,13]. Furthermore, in the early disease scenario (with curative intention), its value for monitoring during treatment has been demonstrated, as well as for detecting relapse and early metastasis, as happened in our patient[12, 13]. Some trials have demonstrated the potential of ctDNA in peripheral blood (both early and metastatic disease) for diagnosis, treatment, and prognosis[14]. There are currently no guidelines or regulatory approvals to monitor response in breast cancer patients[15].

Novel trials has shown that ctDNA has a prognostic value in TNBC, predicting minimal residual disease (MRD) and early relapse after neoadjuvant chemotherapy with curative intent with high specificity[16,17]. ctDNA detection is useful for mo-

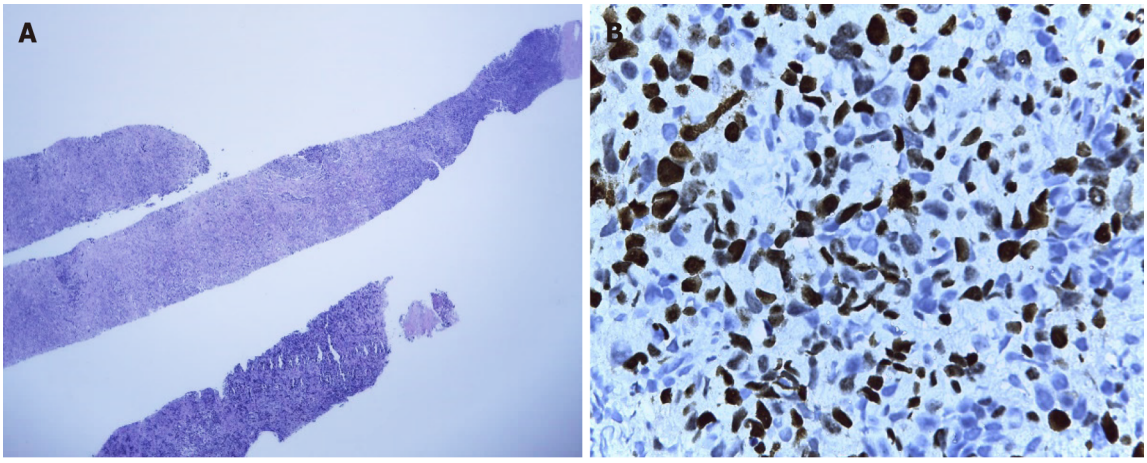


Figure 11 Biopsy of the left axillary node. A: Compatible with metastases of infiltrating primary breast carcinoma; B: Immunohistochemistry in relation to triple-negative breast cancer tumor (ki67: 60%-70%).

nitoring micrometastases or MRD after neoadjuvant chemotherapy and surgery with apparent curative intent in patients with early breast cancer either at a single point (post-surgery) or with serial plasma samples (during follow-up). Consistently, post-surgical levels of ctDNA were found to be predictive of poor prognosis and risk of relapse[16,17]. In one trial, detection of PIK3CA mutation using post-surgical ctDNA predicted disease relapse after 8.1 mo of follow-up[16]. Another trial demonstrated the ability of ctDNA to identify patients at risk of relapse [23/26 patients who relapsed (88.4%) had a history of ctDNA detection with a median time of 10.7 mo before clinical relapse][18]. These results regarding time to early relapse are very similar with our patient. Moreover, the BRE 12-158, a phase II trial presented at the San Antonio Breast Cancer Symposium 2019, showed that the presence of frequent mutations in breast cancer detected by ctDNA and circulating tumor cells are useful to predict recurrence after neoadjuvant chemotherapy in patients with early TNBC. ctDNA was detected in 64% of samples, and TP53 was the most frequent gene. ctDNA was associated with poor distant disease-free survival (DDFS): after 24 mo, the likelihood of DDFS was 25% worse in patients with positive ctDNA (56%) *vs* those with negative ctDNA (81%) (hazard ratio [HR]: 2.99, 95% confidence interval [CI]: 1.38-6.48, $P = 0.0055$). Women with positive ctDNA have 3 times the risk of developing distant disease recurrence compared to those with negative ctDNA (disease-free survival: HR: 2.67, 95%CI: 1.28-5.57, $P = 0.0069$). Furthermore, ctDNA-positive women had a 4.1-fold higher risk of death compared to ctDNA-negative women (overall survival: HR: 4.16, 95%CI: 1.66-10.42, $P = 0.0024$)[19].

Although there is a positive PIK3CA mutation result in this case report, to date, there are no standardized procedures for PIK3CA detection in breast cancer in Peru. Therefore, the application of ctDNA should be based on defined protocols to ensure reproducibility of results, due to mostly undetectable levels of ctDNA in early breast cancer[20]. Finally, it is not known whether the presence of the PIK3CA mutation in peripheral blood and tumor tissue confers a worse prognosis compared to having the mutation in 1 of the 2 samples evaluated (blood, tissue). These findings could increase the interval of control visits or earlier approaches for follow-up to detect local or distant relapse.

CONCLUSION

From the perspective of Personalized Medicine, it is important to characterize the genetic profile of tumors, either in liquid biopsy and/or tumor tissue, in order to detect potential biomarkers and subsequently new targeted therapies. In the case report, the detection of the PIK3CA mutation in a patient with locally advanced TNBC after neoadjuvant treatment and surgery may be associated with an early relapse of disease. Many trials have shown the value of detecting the PIK3CA mutation in liquid biopsy (ctDNA) in patients with TNBC as a potential biomarker for identifying early relapse and disease progression. The detection of PIK3CA mutations might help identify patients at risk of recurrence, and it could also generate closer therapeutic or

follow-up behaviors in order to detect micro metastatic disease/MRD and distant disease.

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REVIEW

Embracing cancer immunotherapy with vital micronutrients

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Abstract

Immunotherapy is now commonly prescribed to cancer patients, but autoimmune-related adverse events are considerable. For severe, life-threatening side effects, cessation of therapy seems unavoidable, let alone intensive medical care required for patching up the adverse events. Even without serious adverse events, the response rates are too low and various combinatory regimens have been tried. However, toxicities are also added on, unless the adjuvant agents have remarkably few side effects. Actually, micronutrients are usually taken by a majority of cancer patients as nutritional support or to boost the immune function, let alone hoping to counteract treatment side effects. Recent studies have shown that combinations of micronutrients exert pleiotropic effects in controlling tumor growth and metastasis by modulating the tumor microenvironment, enhancing gut microbiota immune functions, and providing adjunct nutritional support to micronutrient deficient cancer patients. A higher than recommended dietary allowance micronutrient dose is proposed to reduce the toxic free radicals generated as a result of immunotherapy and tumor metabolism. This is not only helpful for managing treatment side effects but also enhancing treatment efficacy. As micronutrient supplementation is also useful to improve patients' quality of life, prolong survival, and sustain compliance to immunotherapy, further investigations are mandatory.

Key Words: Immunotherapy; Micronutrients; Immune-related adverse events; Vitamins; Tumor microenvironment; Immunonutrition

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Core Tip: Micronutrients in combination may enhance immunotherapy efficacy by

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immunomodulation and minimizing immune-related adverse events, improve acquired immune response through modification of the tumor microenvironment, enhance gut-microbiota immune functions, boost immune-nutrition function, and improve patient outcome.

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INTRODUCTION

It was estimated that 30% to 90% of cancer patients took some form of supplements and micronutrients for immunity support and reducing treatment side effects upon being diagnosed with cancer. Micronutrients such as various vitamins and minerals, especially selenium, zinc, *etc.*, are often consumed without any discussion with their oncologists for fear of being criticized. After all, the role of micronutrients for cancer patients is not generally accepted. Actually, micronutrients such as vitamin C (usually at high dosages) have been used since its discovery in the 1930s not just as a nutritional supplement but also as an anti-microbial agent when there were no potent anti-microbial agents by then[1,2]. Currently, micronutrients are much more often employed by naturopaths and complementary and integrative medical practitioners with or without other modalities to treat chronic diseases, autoimmune disorders, and even cancers[3]. Even in this era of cancer immunotherapy, various immune-related adverse events (irAEs) constitute a real concern. Nevertheless, micronutrients may well be useful for tackling some of these adverse events and even enhance the efficacy, as is being alluded to in this review.

CANCER IMMUNOTHERAPY: IRAES

Checkpoint protein inhibitors (CPIs), including cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) inhibitors and programmed cell death protein 1 pathway/programmed cell death protein 1 ligand (PD-1/PDL-1) inhibitors, are now commonly employed to treat a progressively wider spectrum of cancers with fewer side effects and much better tolerance than classical chemotherapy[4]. Unfortunately, the response rates are low and the immune-related toxicities are considerable[5]. CPIs act by enhancing the immune function of T cells by blocking the connection between PD-1 and PDL-1 and preventing the inhibition of T cells. T cell cytotoxicity then attacks the tumor cells. CTLA-4 blocks the connection between dendritic cells and T cells related to CTLA-4. CTLA-4 removes the inhibition related to dendritic cells on T cells to achieve a cancer-killing effect. Because checkpoints may also regulate autoreactivity, immune checkpoint inhibitor therapy is complicated by irAEs[6]. The mechanisms leading to irAEs are similar to those promoting anti-tumor responses, which involve T and B cell immune modulation and induce autoantibody production [7]. However, the wide range of irAEs associated with immune checkpoint blockade may be diverse and serious. These may well lead to the suspension of the otherwise effective immunotherapy. The irAEs may affect various organs and patients would have multiple side effects. In a study of 78 patients receiving CPIs, 53% developed irAEs with 15% of patients developing more than one complication[4]. Notably, a small number of side effects are life-threatening or require urgent medical attention [8]. Some serious irAEs are colitis, interstitial pneumonitis, myocarditis, pericarditis, arrhythmia, impaired ventricular function, and vasculitis. Neurological complications such as myasthenia gravis, Guillain-Barrie syndrome or peripheral neuropathy, aseptic meningitis, and encephalitis are also documented. Endocrine side effects such as hypothyroidism, hyperthyroidism, adrenal insufficiency, and type I diabetes mellitus, as well as hepatitis, nephritis, autoimmune hemolytic anemia, thrombocytopenia, skin rashes, and bullous dermatoses are also seen[9]. Since many of these side effects are related to similar immunologic actions for the immunotherapy therapeutic effects, the management of such adverse events constitutes a major challenge. Ideally, an efficient

adjuvant drug should be available to enhance cancer immunity whilst alleviating the irAEs[10]; otherwise, irAEs may preclude the continuation of CPIs[8,11]. Currently, medical management of irAEs may often be limited to symptomatic relief with systemic corticosteroids or immunosuppressants together with specialist care. There is a great need for multidisciplinary guidance from different specialties to establish broad-based perspectives in early recognition and management of organ-specific irAEs and to set up management guidelines[12]. Notably, the Society for Immunotherapy of Cancer has set up such a multidisciplinary Toxicity Management Working Group to develop recommendations and initiate treatment protocols for irAEs[11].

ROLE OF VITAL MICRONUTRIENTS IN IMMUNE FUNCTION AND INFECTION

Micronutrients such as vitamins A, D, C, E, B6, and B12, folate, zinc, iron, copper, and selenium are best tailored according to age-related needs[13]. As adequate amounts of these micronutrients are vital for proper immune functioning[14], a high enough dose is necessary for various kinds of immuno-compromised or even the terminally ill[15,16]. According to some studies, micronutrients with the strongest evidence for immune support are vitamin C, vitamin D, and zinc[15,17,18].

Patients with micronutrient deficiencies are prone to various infections and even body dysfunctions due to weakened immune responses to pathogens such as viruses like SARS-CoV-2, the virus that causes COVID-19[19]. Strikingly, micronutrient deficiencies affect about two billion people worldwide[20], contribute to low immunity against infections, and constitute a common cause of immunodeficiency in developing countries[21]. On the other hand, micronutrient supplementation could enhance immune functions and help the body to fight against pathogens and cancers[15,22-24].

CLINICAL IMPACT OF MICRONUTRITION IN CANCER TREATMENT

Since the 1980s, there was abundant epidemiologic evidence that high intakes of fruits and vegetables reduced the risks of most cancers. This may support the concept that micronutrients could play a vital role in cancer prevention[24]. Recent systematic reviews on micronutrients and breast cancer[25] have shown that micronutrient consumption may reduce the incidence rates and/or progression of cancers[24]. Epidemiological and experimental studies showed that the percentage of cancer-related deaths attributable to diet and tobacco was as high as 60%-70% worldwide[26]. For micronutrients, *in vitro* and *in vivo* studies on over 50 human cancer cell lines have demonstrated a good anti-cancer effect being achieved in combinations of micronutrients (rather than the individual compounds). It was also well documented that nutrient combinations exert pleiotropic effects in controlling tumor growth, invasion, and metastasis[16,27-29].

CONTROVERSY OVER USE OF MICRONUTRIENTS IN CANCER THERAPY

Since most micronutrients may also act as antioxidants, some physicians are concerned about possible inhibitory effects on chemotherapy killing actions[30]. On the contrary, there are reliable studies on the beneficial effects of antioxidants and micronutrients for patients during radiation therapy[31,32] and chemotherapy[33,34]. A recent extensive review comprising of 174 peer-reviewed articles and 93 clinical trials with a total of 18208 cancer patients showed that antioxidants have superior potentials in reducing chemotherapy-induced toxicity[35]. The conclusion was that antioxidant supplementation during oncology treatments enhanced chemotherapeutic efficacy and even prolonged patient survival. Moreover, in other studies, when antioxidants were given concurrently with chemotherapy, no interference occurred. Rather, they enhanced the chemotherapeutic effects, and even protected normal tissues and increased patient survivals and therapeutic responses[36,37].

VITAL MICRONUTRIENTS — ROLE IN AMELIORATING IRAES AND ENHANCING IMMUNOTHERAPY

Tumor microenvironment modification

The tumor microenvironment (TME) is largely composed of mesenchymal stem cells, fibroblasts, endothelial cells, adipocytes, and immune cells with an altered extracellular matrix having an acidic and hypoxic composition. TMEs can promote immune tolerance through the secretion of lactate and competing for nutrients between tumor cells and immune cells[38]. Cancer-associated fibroblasts and solid tumors can promote immunosuppression by inhibiting T cell functions and extracellular matrix remodeling[39]. Recent studies have suggested that nutrients available in the TME can influence immunotherapy response and cancer cell metabolic pathways[38,40]. Micronutrients like vitamin C can enhance immune cell functions by modifying the TME by hypoxia-inducible factors[41]. High-dose vitamin C modulates infiltration of the TME by immune cells and delays cancer cell growth in a T cell-dependent manner. Vitamin C enhances the proliferation and maturation of T cells and natural killer cells[42]. It also reduces the formation of neutrophil extracellular traps in the TME, which are related to irAEs due to checkpoint blockade[43]. The combination of high-dose vitamin C and immune checkpoint therapy may potentially enhance the efficacy of immunotherapy for cancer[44].

Vitamin D supplementation also suppresses tumor angiogenesis, progression, and metastasis *via* targeting components of the TME[45]. The active form of vitamin D, $1,25(OH)_2D_3$, regulates stromal cells including tumor-associated fibroblasts, tumor-derived endothelial cells, cancer stem cells, and infiltrating immune cells within the TME to facilitate cancer suppression. Vitamin D also has anti-inflammatory effects within the TME. This leads to the inhibition of proliferation, induction of apoptosis and differentiation, suppression of migration, and autophagic cell death of tumor cells [45]. Taken together, these may reaffirm the anti-cancer potential of vitamin D[46].

Enhancing gut microbiota immune functions

Micronutrient deficiencies have been linked to changes of bacterial species in the human gut microbiota affecting the host regulation of immune responses[47]. The activity of the gut microbiota has significantly contributed to the host immune health and is linked to the development of many diseases including cancer. Therapeutic interventions to optimize microbiota composition to improve immunotherapy outcomes have shown promising results[48,49]. In addition, gut microbiota modulations through micronutrient supplementations could effectively enhance efficacy and relieve or tackle resistance during immunotherapy treatments[50]. Gut microbiota may also activate or repress the host's response to CPIs and potentially modulate resistance to cancer immunotherapy[51]. As vitamin D deficiency has been linked to gut dysbiosis and bowel inflammation, vitamin D may play a significant role in gut microbiome regulation and host immune responses[52]. Moreover, vitamin D supplementation has been shown to increase gut microbial diversity significantly. This is a positive health impact on healthy individuals[53] and cancer patients[54].

Adjunct nutrition support for cancer patients

It was estimated that about 30%-90% of patients believed that they had inadequate diets leading to nutritional deficiencies and poor immune functions; some cancer patients were obviously cachexic. Micronutrient deficiencies do have negative impacts on immunotherapy as the host's immunocompetence is weakened. There is also an increased risk of developing irAEs and a negative impact on the patient's quality of life. Nutritional deficiencies can be reversed early if adjunct micronutrients are given before and during oncology treatments. Some chemotherapy drugs may have side effects of depleting certain micronutrients. This tends to worsen the nutritional deficiency, *e.g.*, cyclophosphamide and paclitaxel can deplete vitamin D by an increased breakdown of calcidiol and calcitriol[55]. A cohort study from the Mayo Clinic has shown a 26% reduction of non-small cell lung cancer mortality with improved quality of life and prolonged survival through micronutrient supplementation[56]. Apparently, immunonutrition has the potential to modulate the activity of the immune system by interventions with specific nutrients. It may be applied with immunotherapy to improve immune functions, modulate the acquired immune response, decrease treatment toxicity, and enhance patient outcomes[57]. Micronutrients such as selenium, vitamin C, and vitamin D (at high doses) have been found to be effective and safe for patients undergoing oncological intervention[16,55,58,59].

Protecting normal healthy cells

Immunotherapy-associated irAEs include autoimmune reactions, cytokine release syndromes, and vascular leak syndrome. These vary depending on the type of immunotherapy and the specific mechanism of action. Cytokines such as high-dose IL-2 will lead to capillary leakage and a sepsis-like syndrome or multi-organ failure[60]. CPIs disinhibiting T cell anti-tumor action can lead to a distinct constellation of organ-specific inflammatory side effects or irAEs[12].

Vitamin D and zinc have been known for balancing immune functions through the prevention and treatment of autoimmune diseases[61]. Several observational studies have shown that vitamin D deficiencies increased the risk of autoimmune diseases such as type I diabetes, systemic lupus erythematosus, inflammatory bowel disease, Hashimoto's thyroiditis, multiple sclerosis, psoriasis, and rheumatoid arthritis[62,63]. Vitamin D supplementation is found to be beneficial to prostate, breast, and colorectal cancers and melanoma patients during treatment[64].

Vitamin B12 supplements may reduce the direct toxic side effects of immunotherapy as vitamin B12 is required for red blood cell synthesis, neural functions, and reduction of the severity of drug-induced peripheral neuropathy[65]. Vitamin B12 has been added as a supplement to pemetrexed and cisplatin chemotherapy agents, as used in pleural mesothelioma and non-small cell lung cancer. This was allegedly because of its folate similarity and inhibition of purine and pyrimidine synthesis[66]. Vitamin B12 effectively reduced the toxic side effects of the main chemotherapy.

Vitamin C is concentrated in most immune cells which support essential immune functions such as enzyme cofactors for Fe- or Cu- containing oxygenase. This regulates cell metabolism, epigenetics, growth, survival pathways, and even stem cell phenotypes[42]. High-dose intravenous vitamin C has been found to be useful as an adjunct to interleukin-2 immunotherapy to reduce capillary leakage, systemic complement activation, and a non-specific rise in inflammatory mediators such as TNF-alpha and C-reactive proteins by protecting the endothelium from inflammation [67]. High-dose intravenous vitamin C may also reduce cytokines which cause tumor angiogenesis and inflammation in cancer patients[68].

Vitamin D deficiency has been linked to autoimmune diseases[63] such as psoriasis, vitiligo[69,70], autoimmune thyroid diseases, Hashimoto's thyroiditis, and postpartum thyroiditis[71]. Vitamin D decreases the expression of various cytokines that cause vitiligo and other autoimmune disorders by preventing the destruction of melanocytes [69]. Oral vitamin D3 has been reported to be effective for improving the levels of epidermal keratin in psoriatic patients and to improve the treatment outcome with topical dithranol, PUVA (psoralen and ultraviolet A, a light therapy for skin diseases), and oral etretinate and hydroxyurea therapy[72]. A pilot study with prolonged supplementation of high dose vitamin D has improved the clinical course of vitiligo and psoriasis[73]. Melanoma patients often present with cutaneous lesions such as vitiligo, representing an autoimmune disorder with progressive destruction of melanocytes[74]. Dermatologic side effects such as vitiligo and leukoderma are often seen in melanoma patients who are on PD-1 inhibitors (up to 10%, more for ipilimumab)[75]. Notably, irAEs affect all organ systems and most commonly the skin (pruritus, rash, and vitiligo), the gastrointestinal tract (enterocolitis), the liver (hepatitis), and the endocrine system while less commonly involve the neurological system. The gastrointestinal tract, liver, lung, and skin are actually maintained in an immunologically quiescent state, which may explain the vulnerability of these organs for the development of irAEs[6].

MICRONUTRIENTS: VENTURING TO REDUCE AUTOIMMUNE-RELATED IRAES

Interestingly, a recent cohort study has shown that vitamin D supplementation could reduce the risks of CPI-induced colitis by as much as 65%[76]. As CPI-induced colitis is an irAE that is basically autoimmune-related, such micronutrients as vitamin D may also reduce the risks of other CPI-induced and autoimmune-related irAEs. As alluded to above, vitamin D deficiency is rather closely linked with autoimmune disorders, let alone vitamin D administration may be beneficial. Hence, it would appear highly worthwhile to look at the prospects of such micronutrients in managing autoimmune-related disorders. There may be a potential role of micronutrients in preventing irAEs induced by CPIs. Currently, CPIs do have considerable autoimmune-related irAEs. For instance, the phase 2 KEYNOTE-224 trial of pembrolizumab for advanced hepatocellular carcinoma patients who have been treated previously with sorafenib saw

considerable adverse events[77]. In that trial, treatment-related adverse events occurred in 73% of 104 patients. Most of the more serious adverse events were immune-related. Naturally, serious adverse events may well lead to dropouts or suspension of the immunotherapy, defeating the whole purpose of such a valuable modality of treatment. Apparently, it would be worthwhile to examine whether vitamin D or zinc really has beneficial effects on the management of autoimmune disorders. If so, it may support the feasibility of using these micronutrients prospectively to reduce the autoimmune-related irAEs of CPIs. If some simple measures could prevent or reduce such adverse events, it would be most helpful. More cancer patients may then be able to benefit from CPIs. Before that could ever happen, one could start by scrutinizing how effective are these micronutrients, especially vitamin D and zinc for the management of autoimmune-related disorders. Table 1[78-85] shows selected trials of zinc and vitamin D on autoimmune-related disorders.

Notably, the 3rd study listed in Table 1 involved a combination of zinc and vitamin A supplementation that had been shown to improve serum apoprotein A-1 and apoprotein B levels and the apoprotein B/proprotein A-1 ratio in patients with type 1 diabetes mellitus (T1DM). In fact, the deficiency of vitamin A would mainly involve an impaired transport mechanism of vitamin A from its hepatic storage to the target sites [86]. As insulin therapy would reverse this impairment, the replacement of vitamin A may not be crucial for controlling T1DM. Hence, the beneficial adjuvant effect of the combination of zinc and vitamin A for T1DM was more likely to be due to zinc than vitamin A. Moreover, from Table 1, three studies had involved T1DM cases of recent onset (studies 4, 5, and 6). Apparently, the adjuvant role of micronutrients for T1DM cases of recent onset may be more effective. Possibly, the fact that a vitamin D analog could benefit recent-onset T1DM may suggest that it would be useful to prevent an irAE that involves the beta cells of the pancreas.

Moreover, as micronutrients are but adjunctive treatment modalities, for demonstrating their effectiveness would also depend largely on the main modalities of treatment. In case that there is a significant difference in the effectiveness of those main modalities of treatment between the study groups, then the effectiveness of the adjunctive modalities of treatment would be difficult to demonstrate. Another highly relevant factor is the distribution of genetic predispositions between various groups of the study population. As to balance very evenly the genetic predispositions among the groups is not done easily or not done at all, the effect of such an imbalance between the groups would naturally affect the results[87]. Thus, incidental negative trial findings of micronutrients should not be taken as definitive proof that micronutrients are not useful.

Lastly, even the diet may affect autoimmunity. It was reported that heavy metals like mercury[88] might be incriminated. Chronic exposure to low levels of methylmercury (organic) and inorganic mercury was common among 1352 female subjects 16 to 49 years of age from the US National Health and Nutrition Examination Survey. Probably, the mercury was from consuming fish and even the slow disintegration of dental amalgams. Also, 16% of subjects were antinuclear antibody (ANA) positive. Hair and blood mercury levels were associated with ANA positivity. As ANA is closely related to autoimmune disorders, methylmercury exposure was deemed to be associated with subclinical autoimmunity among subjects and autoantibodies may even predate the onset of clinical diseases by years.

Taken together, several factors may affect the effectiveness of vitamin D and zinc on autoimmune disorders. When trials were performed on such micronutrients, it was challenging to balance evenly all the relevant factors among different arms of those studies. As such, results can be rather variable but may not reflect the true effectiveness of these micronutrients. Thus, negative clinical trial results should not be taken at their face value. After all, all these adjuvants have to act together with other more specific agents before exerting their effects. Moreover, the duration of onset of the autoimmune-related disorders may also be highly relevant. It is also possible that such adjuvant agents are most effective for prevention rather than treatment. In any case, these micronutrients should be further investigated thoroughly for their ability of preventing or reducing early autoimmune-related irAEs induced by CPIs. This is especially so as they have an excellent safety profile, are easily taken and eminently affordable.

Actually, cancer patients who are also suffering concurrently from immune disorders are routinely precluded from receiving any CPI, even if they are already on specific drugs for their autoimmune disorders. This is because of the fear of exacerbating their autoimmune symptoms once CPIs commence. If more studies can be done on vitamin D and zinc on their ability to prevent exacerbation of autoimmune disorder symptoms, one may know how effective these can prevent such autoimmune-related

Table 1 Selected trials on the effect of zinc and vitamin D on autoimmune related disorders

No.	Autoimmune disorder	Agent	Dose	Period	Trial type	Benefit	Year
1	MS	Cholecalciferol	50000 IU/wk	12 mo	R, C, DB	Decreased incidence rate of demyelination plaques, reduced progression risk	2013[78]
2	RA	ZnSO4	220 mg/3×/d	12 wk + 12 wk	C then O	Decreased joint swelling, stiffness, walking time	1976[79]
3	T1DM	ZnSO4 + vit A	10 mg/d + vit A 25000 IU	12 wk	R, C, DB	Increased serum apo A1; decreased apo B/Apo A1 ratio	2010[80]
4	T1DM (RO)	Alpha-calcidol	10 IU/1-2×/d	6 mo	R, C, B (prtps)	FCP higher; lower requirement of insulin	2013[81]
5	T1DM (RO)	Cholecalciferol	2000 IU/d	18 mo	R, C, DB	Protective immunologic effect; slow decline of residual β-cell function (serum FCP and SCP levels)	2012[82]
6	T1DM (RO)	Cholecalciferol	70 IU/kg/d	12 mo	R, C, DB	Improved the suppressive capacity of Tregs	2015[83]
7	PS	Zinc pyrithione topical 0.25% in an emollient base	2×/d	3 mo	R, C, DB	Decreased plaques/PASI score	2011[84]
8	SLE	Vit D	50000 IU/wk	24 wk	R, C, DB	Decreased disease activity parameters; reduced fatigue	2016[85]

Apo: Apoprotein; B: Blind; C: Controlled; DB: Double blind; FCP: Fasting C-peptide; MS: Multiple sclerosis; O: Open; PASI: Psoriasis area and severity index; prtps: Participants; PS: Psoriasis; R: Randomized; RA: Rheumatoid arthritis; RO: Recent onset; SCP: Stimulated C-peptide; SLE: Systemic lupus erythematosus; T1DM: Type 1 diabetes mellitus; Treg: Regulatory T cells; Vit: Vitamin.

irAEs of CPIs. Hopefully, these unfortunate cancer patients suffering from two major disorders may then benefit from CPIs. Even those patients without any pre-existing autoimmune disorders may also benefit from reduced autoimmune-related irAEs upon commencing CPIs. Their autoimmune-related irAEs may be reduced by micronutrients and those unplanned suspensions of CPIs are avoided. Even for those who already have such unfortunate suspensions, such micronutrients might still contribute to a more successful rechallenging program. After all, if there are no other realistic options than CPIs, the threat to life is actually higher for uncontrollable cancers than autoimmune-related irAEs.

Immunomodulating micronutrients enhances immunotherapy

Vitamin A, beta-carotene, folic acid, vitamin B12, vitamin C, vitamin D, riboflavin, iron, zinc, and selenium may all have immunomodulating functions and could enhance the immune response rates of immunotherapy and even reduce irAEs[89]. They play an important role in reducing oxidative stress in diseases and cancers. Vitamin A supplementation improves levels of IgA immunoglobulin and CD40 ligand-activated IgG and reduces inflammatory cytokine levels[90]. Vitamin E as a potent antioxidant would reduce inflammation by modulating T cell function and downmodulating prostaglandin E2 in patients[91]. Vitamin C improves immune functions by supporting natural killer cell activities, lymphocyte proliferation, and chemotaxis, stimulates dendritic cells to secrete interleukin-12, and activates T and B cell functions[42]. High-dose vitamin C not only enhances the cytotoxic activity of CD8 T cells but also enhances immunotherapy by co-operating with immune checkpoint therapy in several cancer types[44]. Vitamin B12 deficiency has been linked to low lymphocyte counts, impaired NK cell function, decreased CD8+ cells, and impaired immune functions. Eventually, the raised CD4/CD8 ratio[92] would be potentially reversible by oral or intramuscular B12 injections. Vitamin D [1,25-$(OH)_2D_3$] binds to the vitamin D receptor of both the antigen-presenting cells (APC), dendritic cells, and T lymphocytes so as to exert its indirect and direct effects on T lymphocytes. The latter effect on the T lymphocytes is a change towards a more tolerogenic (capable of producing immunological tolerance) state with induction of T helper-2 (Th2)-lymphocytes and regulatory T lymphocytes (Tregs), together with a downregulation of the pro-inflammatory Thelper-1 (Th-1)-lymphocytes, Thelper-17 (Th-17)-lymphocytes, and Thelper-9 (Th9)-lymphocytes][93]. Notably, vitamin D suppresses T cell prolif-

eration and then results in a shift from a Th-1 to a Th-2 development, inhibition of Th-17 cell development, and also facilitation of T regulatory cells with an arrest of cytotoxic T lymphocyte infiltration as well as increased $CD4^+CD25^+$ Tregs[94]. Lastly, vitamin D inhibits inflammatory cytokine production by monocytes, and suppresses dendritic cell differentiation and maturation. This helps to maintain tolerance and would also promote protective immunity[95].

DISCUSSION

Micronutrients are closely associated with the body's immune functions; a micronutrient deficient subject will have poor immune status and be prone to infections and even cancer development. Immunotherapy is emerging as an important adjunct oncology modality of treatment. The key to success is dependent on a good host's immune response to tackle cancers. The target of immunotherapy is killing the cancer cells with minimal collateral damages and leaving the body's immune system intact. Even though cancer immunotherapy provides a better option than chemotherapy, achieves higher success rates, and causes less marrow depression, it has considerable limitations. More than half of treated patients develop irAEs[4], let alone only a minority of cancer patients respond well to immunotherapy. Moreover, a minority of irAEs can be serious and even fatal. To overcome these limitations, supplementation of vital micronutrients to immunotherapy patients seems to be the simplest and the most pragmatic way of reducing such irAEs. Micronutrients have been used successfully in conventional oncology to reduce treatment side effects, enhance therapy efficacy, prolong survival, and improve quality of life[25,27,28,59,96]. For immunotherapy, despite less clinical experience, similar biophysiological mechanisms may also work when micronutrients are added to immunotherapy. Realistically, micronutrients may well offer comparable benefits to immunotherapy patients by strengthening the immune cell functions, enhancing tumor-killing effects, and reducing or preventing treatment complications[55].

Notably, micronutrient deficiency in one particular nutrient is rather difficult to diagnose and clinical symptoms may not be obvious, let alone overlapping effects with other clinical conditions. Thus, for best results, micronutrients as an adjunct oncology therapy should be given prospectively and in combination with the main treatment[15,97].

Unfortunately, there are no standard micronutrient supplementation protocols for immunotherapy patients. Despite some negative findings[37,98], a general consensus could still be built on the effectiveness of known positive trials and the remarkable safety profile of micronutrient therapy. After all, negative trials may well be due to various related factors and the imbalance of trial participants in various arms, as has been discussed in great detail. Moreover, as the antioxidant effect of micronutrients has already been proven to be not a concern, some studies advocate using higher than the recommended dietary allowance doses of micronutrients in combination for cancer patients to achieve optimal benefits[44,59,96,99]. A higher dose of micronutrients offering greater antioxidant effects may better tackle free radicals generated during immunotherapy and also enhance host immune function[15,100]. Importantly, future oncology research should be directed towards investigating the effects of different groups of micronutrients in combination with the main oncology modalities of treatment for different cancer types so as to delineate the optimal micronutrient regimens for immunotherapy.

CONCLUSION

Micronutrients used to play an active role in the past. High-dose vitamin C has been administered for viral infections before the debut of more specific agents; vitamin D has also been used for treating some autoimmune disorders before more specific agents are now available for such disorders. Currently, these and similar micronutrients should be investigated actively to better define their definitive adjuvant role in the era of cancer immunotherapy. Actually, micronutrients play a pivotal role in maintaining good immune cell functions and would also play an integral role in the defense against infectious agents and even cancers. Adequate amounts of micronutrients during immunotherapy have been shown to have the potential of enhancing immunotherapy efficacy, reducing irAEs, improving patients' quality of life, prolonging survivals, and even sustaining the best treatment compliance. As the use of

micronutrients as adjuvants for oncology treatments is still in its infancy, many more studies are required to explore the full potential of such safe, convenient, and affordable agents.

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REVIEW

Metastatic disease to the liver: Locoregional therapy strategies and outcomes

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Abstract

Secondary cancers of the liver are more than twenty times more common than primary tumors and are incurable in most cases. While surgical resection and systemic chemotherapy are often the first-line therapy for metastatic liver disease, a majority of patients present with bilobar disease not amenable to curative local resection. Furthermore, by the time metastasis to the liver has developed, many tumors demonstrate a degree of resistance to systemic chemotherapy. Fortunately, catheter-directed and percutaneous locoregional approaches have evolved as major treatment modalities for unresectable metastatic disease. These novel techniques can be used for diverse applications ranging from curative intent for small localized tumors, downstaging of large tumors for resection, or locoregional control and palliation of advanced disease. Their use has been associated with increased tumor response, increased disease-free and overall survival, and decreased morbidity and mortality in a broad range of metastatic disease. This review explores recent advances in liver-directed therapies for metastatic liver disease from primary colorectal, neuroendocrine, breast, and lung cancer, as well as uveal melanoma, cholangiocarcinoma, and sarcoma. Therapies discussed include bland transarterial embolization, chemoembolization, radioembolization, and ablative therapies, with a focus on current treatment approaches, outcomes of locoregional therapy, and future directions in each type of metastatic disease.

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Core Tip: Locoregional percutaneous catheter-directed approaches have been associated with better tumor response, improved disease-free and overall survival, and decreased morbidity in metastatic disease to the liver compared to standard treatment. This review explores recent advances in liver-directed therapies for metastatic liver disease from primary colorectal, neuroendocrine, breast, and lung cancer, as well as uveal melanoma, cholangiocarcinoma, and sarcoma. Therapies discussed include bland transarterial embolization, chemoembolization, radioembolization, and ablative therapies, with a focus on current treatment approaches, outcomes of locoregional therapy, and future directions in each type of metastatic disease.

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INTRODUCTION

Metastatic disease to the liver is the most common malignant liver condition and a major cause of cancer-related morbidity and mortality[1]. While colon cancer represents the most common metastatic disease to the liver, other common primary tumors include lung and breast adenocarcinomas, neuroendocrine tumors, melanomas, and sarcoma[2]. Regardless of primary tumor type, liver metastasis generally represents advanced disease, and is typically associated with poor prognosis[3]. The goals of therapy at this stage are often palliative, but there is a growing interest in differentiating between oligometastatic disease characterized by limited metastasis from more widespread metastatic disease, as these classifications may carry prognostic value[4-7]. In some cases, curative treatment has been demonstrated in oligometastatic disease, encouraging aggressive local treatment in appropriate patients[8]. Traditional management options for patients with metastatic disease to the liver include surgical resection and systemic chemotherapy. However, the percent of patients who present with disease amenable to surgery ranges from 25% to less than 10% depending on the primary tumor[9,10]. While significant advances in complex liver surgery have been made over the past several decades, liver resections are nevertheless still associated with major morbidity and mortality[11]. These risks must be carefully balanced with the evidence for a survival benefit especially in the setting of metastatic disease[12].

On the other hand, most patients with liver metastases have unresectable disease, either because of anatomical limitations, presence of extrahepatic disease, or absence of evidence establishing a survival benefit for resection. Fortunately, novel liver-directed strategies are being used to downstage tumors for curative resection, reduce symptoms, and provide better tumor control[13-15]. In the last ten years, locoregional therapies in metastatic liver disease have demonstrated comparable outcomes with fewer side effects than current standards of care, leading to formal incorporation into treatment algorithms as first-line, adjunctive, or second-line therapy for various tumor types[16-19]. The development of new image-guided techniques and enhanced targeted pharmaco- and radiotherapeutics promise to improve upon the impressive tumor response, progression free survival (PFS), and overall survival (OS) rates that these therapies have already demonstrated. This review examines the recent advances in locoregional therapy for metastatic disease to the liver including transarterial embolization (TAE), transarterial chemoembolization (TACE), transarterial radioembolization (TARE), and ablative therapies in major cancer types.

LOCOREGIONAL THERAPIES

TAE, TACE, and TARE

Catheter-directed locoregional therapies are based on the principle that liver tumors recruit their blood supply from the hepatic artery, while hepatic parenchymal cells are primarily supplied by the portal vein. In this way, local therapies such as TAE, TACE, and TARE can be targeted to tumor cells while minimizing damage to normal liver tissue[20]. In all cases, the key branches of the hepatic artery supplying the tumor are identified before the introduction of embolic agents, chemotherapy, or radiotherapy to prevent non-target embolization[21] (Tables 1 and 2).

In TAE, particulate or liquid embolic agents are administered, resulting in cellular membrane disruption and ischemic cell death. Similarly, in TACE, tumor vessels are occluded, with the added benefit of local delivery of chemotherapeutic agents. In the conventional approach (c-TACE), a lipiodolized chemotherapeutic is introduced, followed by the embolic agent. More recently, drug-eluting beads (DEB-TACE) have been used as both chemotherapeutic and embolic agents allowing for the sustained release of chemotherapy with greater standardization compared to c-TACE. The most common complication of these procedures is postembolization syndrome (PES), which presents as self-limiting right upper quadrant pain, nausea, fever, and elevated liver function tests. PES is attributed to tumor necrosis and tissue ischemia and full recovery within seven to ten days is typical. Other risks include hepatic decompensation, renal injury, biliary injury, infection, and non-target embolization[21].

In a similar fashion, TARE uses 30-micron beads that have been embedded or coated with a radioisotope of yttrium (*i.e.*, ^{90}Y). Once introduced, ^{90}Y undergoes beta-decay causing radiation-induced damage to cellular DNA repair mechanisms and ultimately cell death. One benefit of TARE over TACE is that is can be delivered in the outpatient setting[22]. Unique complications of TARE include radioembolization-induced liver disease (REILD) and post-radiation syndrome. REILD is seen in up to 20% of patients treated with TARE and defined by jaundice and ascites that persist 1-2 mo after treatment without evidence of obstruction or tumor progression. In contrast, post-radiation syndrome is a set of non-specific symptoms including fatigue, nausea, anorexia, and fever generally requiring supportive management[23].

To assess response, follow up imaging and laboratory investigations are conducted 4-6 wk later, and every 3-6 mo thereafter to evaluate treatment success and monitor disease progression. Laboratory evaluation includes tumor markers such as CEA and CA19-9 for colorectal and cholangiocarcinoma[24] or chromogranin, pancreatic polypeptide, or pancreastatin for neuroendocrine tumors[25,26]. To evaluate response with imaging, multiple criteria have been created. One way to evaluate response is by assessing changes in tumor size with contrast-enhanced CT or MRI imaging, which is the basis of one commonly used set of response criteria, termed Response Evaluation Criteria in Solid Tumors (RECIST)[27]. Similarly, PET Response Criteria in Solid Tumors (PERCIST), was developed to measure changes in radiotracer uptake on positron emission topography imaging[28]. The development of novel therapies has led to the development of tumor-specific imaging criteria including the modified RECIST (mRECIST) criteria for hepatocellular carcinoma, Modified CT Response Evaluation (Choi) Criteria for gastrointestinal stromal tumors, and the European Association for Study of the Liver (EASL) criteria, among others[28-31]. These criteria were developed to take into account functional changes seen on imaging, such as contrast enhancement or density, when using therapies that may not lead to radiographic reductions in tumor size[32].

Ablative strategies

Ablative techniques include radiofrequency ablation (RFA), microwave ablation (MWA), cryoablation (CA), irreversible electroporation (IRE), laser-induced interstitial thermotherapy (LITT), and high-intensity focused ultrasound (HIFU). Commonly used ablative techniques can be performed *via* intravascular approach, percutaneously, or in conjunction with surgical resection. While RFA is generally the most commonly studied method, alternate techniques such as MWA have become increasingly popular. In contrast, the use of CA has declined due to its increased post-procedure morbidity and local recurrence rates compared with other methods[33,34]. CA is associated with a number of other unique adverse effects including myohemoglobinuria leading to acute renal failure, cardiac dysrhythmias, and cryogenic shock, a cytokine-mediated syndrome of multi-organ failure, severe coagulopathy, and disseminated intravascular coagulation[21,35]. IRE, LITT, and HIFU remain less well-studied modalities for treatment of liver metastases but have demonstrated promise in clinical

Table 1 Summary of locoregional therapy options for metastatic disease to the liver

Modality	Techniques	Risks
TAE	Particulate or liquid embolic agents	PES, liver abscess, liver biloma, liver failure
TACE	Conventional emulsified chemotherapeutic agent (c-TACE) or drug-eluting beads (DEB-TACE)	PES, liver abscess, liver biloma, liver failure
TARE	Yttrium-90 radioisotope loaded on microspheres	REILD, PRS, liver failure, liver abscess, liver biloma
Ablation	Radiofrequency, microwaves, laser, cooling, alternating and direct current	PAS, bleeding, damage to surrounding structures

PES: Post-embolization syndrome; REILD: Radioembolization-induced liver disease; PRS: Post-radioembolization syndrome; PAS: Post-ablation syndrome; TAE: Transarterial embolization; TACE: Transarterial chemoembolization; TARE: Transarterial radioembolization.

Table 2 Applications and outcomes of locoregional therapies by tumor type

Modality	Applications and outcomes
TAE	First-line for unresectable symptomatic well-differentiated NELM refractory to medical therapy [19]
	Improved OS and PFS *vs* first-line chemotherapy in unresectable CRLM[39,40]
TACE	Comparable tumor response and OS *vs* first-line chemotherapy in neoadjuvant setting for CRLM [41]
	Improved OS and tumor control when used as adjunctive therapy in BCLM[42-44]
	Comparable overall survival to systemic chemotherapy in UMLM[45,46]
	In IHC, DEB-TACE and chemotherapy have comparable OS[47] and DEB-TACE improves OS when added to chemotherapy[48,49]
	TARE with first-line chemotherapy offers a survival benefit in CRLM[50], IHC[51]
TARE	Provides survival benefit in CRLM after failure of two lines of chemotherapy[52]
	TARE plus chemotherapy improves downstaging *vs* chemotherapy alone in CRLM[13,53], IHC[51]
	Increases OS in unresectable CRLM compared to chemotherapy alone[54]
Ablation	RFA[55] and MWA[56] have comparable OS to surgical resection in CRLM
	RFA with resection has comparable OS to two-stage hepatectomy in CRLM[57], NELM[58]
	Fewer adverse events, longer PFS, and comparable OS *vs* resection in BCLM[12,59]

NELM: Neuroendocrine cancer with liver metastasis, CRLM: Colorectal cancer with liver metastasis, BCLM: Breast cancer with liver metastasis, UMLM: Uveal melanoma with liver metastasis, IHC: intrahepatic cholangiocarcinoma, OS: Overall survival, PFS: Progression-free survival; TAE: Transarterial embolization; TACE: Transarterial chemoembolization; TARE: Transarterial radioembolization.

studies[21] (Tables 1 and 2).

RFA uses a locally introduced electrode to emit radiofrequency alternating current to generate thermal energy that results in tumor necrosis. It is most effective in small tumors (< 3 cm) and in metastases with fewer lesions and is less effective in tumors located close to the hilum and large blood vessel due to the heat sink effect of flowing blood[36,37]. To combat size limitations, multiprobe stereotactic RFA is a technique that shows promise for hepatic tumors up to 8 cm[38]. MWA similarly uses a locally introduced antenna to generate an electromagnetic field that aligns nearby water molecules, producing thermal energy. In contrast to RFA, MWA achieves target temperatures faster over a larger area, produces more uniform heating zones, and is less susceptible to heat sink effects. Further, MWA has the ability to perform multiple ablations simultaneously[39]. The newest form of ablation is irreversible electroporation (IRE). In contrast to thermal ablation techniques, IRE uses high-voltage electrical current to create permanent nanopores in the cell membrane, leading to apoptosis[40]. Serious complications common to ablative strategies include bleeding, damage to surrounding organs such as the diaphragm, GI tract, and gall bladder, and a self-limiting post-ablation syndrome (PAS) that presents with the same symptoms as

PES[41,42].

COLORECTAL CARCINOMA

Introduction to colorectal liver metastasis

Colorectal liver metastasis (CRLM) is the most common type of liver malignancy, and over one half of patients with colorectal cancer will develop metastasis to the liver[43]. Interestingly, in left-sided colorectal cancer, liver metastasis is less extensive with better overall survival. In contrast, metastasis to the liver in right-sided colorectal cancer is more extensive with worse survival[44]. Surgical resection remains the first-line treatment for CRLM, but only about 25% of patients are surgical candidates[9]. In recent years, there has been a substantial increase in evidence supporting local therapies for surgically untreatable CRLM. Current guidelines now support the use of local therapies after neoadjuvant systemic chemotherapy has failed to successfully downgrade a surgically unresectable tumor[45]. Currently, ablation is being explored as an alternative to surgical management in select cases of CRLM. Additionally, the benefits of TARE and TACE in conjunction with systemic chemotherapy and ablation are being actively explored.

TACE in CRLM

While c-TACE with doxorubicin is commonly employed for primary hepatocellular carcinoma, doxorubicin does not have the same efficacy against colorectal metastasis. In response, there has been growing interest in DEB-TACE with irinotecan (DEBIRI), a chemotherapeutic used primarily in the treatment of colorectal carcinoma. In 2012, Fiorentini *et al*[46] conducted the first randomized clinical trial on DEBIRI *vs* FOLFIRI (systemic irinotecan, fluorouracil, and leucovorin), which demonstrated the superiority of DEBIRI in terms of overall survival (22 *vs* 15 mo) and progression free survival (7 mo *vs* 4 mo). Metanalysis of studies since then have demonstrated an average tumor response rate of 62%, with median OS of 18 mo with DEBIRI, and 33 mo when DEBIRI is combined with FOLFOX[47]. DEBIRI has additionally been explored as a neoadjuvant therapy: PARAGON II demonstrated that a single treatment of DEBIRI was comparable to systemic neoadjuvant chemotherapy in terms of tumor response and overall survival[48]. Interestingly, one study on DEBIRI examined outcomes stratified by left-sided or right-sided primary colorectal cancer and found that left-sided colorectal cancer was associated with median OS of 33 mo, while median survival in right-sided colorectal cancer was only 17 mo[49].

TARE in CRLM

Transarterial radioembolic therapy with yttrium-90 has shown a survival benefit in unresectable CRLM. The MORE trial, a retrospective study of 606 patients with unresectable CRLM refractory to one or more lines of chemotherapy, demonstrated that treatment with TARE resulted in median OS of 10 mo[50]. SIRFLOX, a phase III trial, was designed to compare standard systemic chemotherapy (FOLFOX +/- bevacizumab) with systemic chemotherapy plus TARE. Results from this trial showed that the addition of TARE was associated with comparable survival, longer progression free survival, and better tumor response rates in the liver[51]. Further, adding TARE to systemic chemotherapy is associated with less viable tumor tissue after treatment and greater gains in resectability of primarily unrespectable tumors than systemic chemotherapy alone[13,52]. Differences in outcomes between right and left-sided colorectal cancers have been demonstrated in TARE. Of note, TARE added to first-line FOLFOX was associated with a 4.9 mo increase in median OS compared to chemotherapy alone, a difference that was not seen in patients with left-sided primary tumors[53]. Further phase III trials are currently underway to assess the benefit of adjunctive TARE with second-line chemotherapy in CRLM[54].

Ablation in CRLM

Ablation has been long studied in CRLM and demonstrates comparable outcomes to resection when used for small tumors (< 3 cm) with appropriate margins (> 5 mm) [37]. Phase II trials on unresectable colorectal liver metastases revealed that the addition of RFA to systemic chemotherapy increased OS at eight years to 35.9% from 8.9% with chemotherapy alone, with a median OS of 45.6 mo[55]. Recent work has demonstrated the non-inferiority of RFA and MWA for treatment of small resectable liver metastasis[56-58]. These results led to the ongoing COLLISON trial, a rando-

mized, controlled phase III trial comparing overall survival in RFA *vs* surgery in resectable CRLM < 3 cm. Initial discussion suggests there may be a particular role for radiofrequency ablation in small but deep-seated tumors, which would require major hepatectomy using traditional surgical approaches[45]. If shown to be non-inferior, there would be many benefits to adopting a minimally invasive approach like ablation, including decreased morbidity and mortality, length of hospital stay and recovery time. Regarding MWA, recent metanalyses suggest superiority over RFA for resectable CRLM: MWA was found to have similar adverse effect profile and may be associated with increased overall and disease-free survival[59].

Future directions

The five-year survival rate for CRLM has been increasing in recent decades, in part due to locoregional approaches that have enabled downstaging and curative resection in previously unresectable patients. With the recent therapeutic options like DEBIRI and the further development of ablative therapies such as MWA, which can be combined with various chemotherapy regimens, these outcomes will hopefully continue to improve. Other therapies currently under investigation include new ablative techniques like irreversible electroporation[45], for which a phase II trial is currently underway (NCT02082782)[60]. While the role for interventional liver-directed techniques continue to expand, additional research is needed regarding the application of these therapies in an adjuvant setting to improve the multidisciplinary care of CRLM and reduce recurrence rates.

NEUROENDOCRINE TUMORS

Introduction to neuroendocrine tumors with liver metastasis

Neuroendocrine tumors (NETs) are a diverse group of neoplasms that arise from neuroendocrine cells in various parts of the body. They are primarily classified by histology and are generally separated into two groups: indolent, well-differentiated tumors, and more aggressive, poorly differentiated carcinomas. NETs can also secrete hormone peptides, resulting in systemic syndromes. The liver is the most common site of metastasis and the majority of patients with metastatic NETs have neuroendocrine liver metastasis (NELM). Treatment options include surgery, locoregional therapies, chemotherapy, somatostatin analogs, and liver transplant in select patients. After failure of somatostatin analogs, resection is the preferred method of treatment in liver-predominant disease, but curative resection is only possible in 10%-25% of patients and recurrence occurs in 50%-95% of patients[61,62]. Ablation can be used as curative therapy and for downstaging of previously unresectable disease. In patients who are not surgical candidates, typically due to bilobar multifocal disease, locoregional therapies including TAE, TACE, and TARE are the preferred approach for tumor control and management of carcinoid symptoms[62]. Even in the absence of complete disease eradication, locoregional therapies can achieve complete remission of symptoms due to hormone peptide secretion.

TAE and TACE in NELM

TAE and TACE are the preferred therapies for well-differentiated, unresectable, liver-dominant NELM with symptoms that are refractory to medical therapy[19]. In a direct comparison, TAE and TACE were associated with similar outcomes, but TAE was associated with fewer adverse effects than TACE[63]. Dermine *et al*[61] pooled the results of 25 retrospective studies (1986-2017) that examined TACE in NELM and found a progression-free survival (PFS) of 18.5 mo with median OS of 34.5 mo. Measures of response rate were variable, but overall the morphological response rate was 49%, with an additional 27% showing tumor stabilization[61]. In a more recent retrospective study, 197 patients with NELM treated with TACE demonstrated a 96% response by RECIST criteria with a median OS of 35.9 mo and PFS of 15.9 mo[14]. In a comparison between c-TACE with cisplatin, mitomycin C and doxorubicin and DEB-TACE with doxorubicin, c-TACE was associated with higher symptomatic response (47% *vs* 30%) but a higher rate of post-embolization and LFT elevations[64]. Currently, there is an ongoing prospective randomized trial comparing TAE, c-TACE, and DEB-TACE which recently closed its DEB-TACE arm based on initial safety data[65].

TARE in NELM

One analysis which pooled 15 retrospective studies of NELM treated with TARE from

2008 to 2016 found a median symptom response of 89.5% (range: 55-100%), median response rate of 51% (range: 12%-73%) by RECIST criteria, PFS of 10 mo (range: 9-11 mo), and median OS of 28.5 mo (range: 14-70 mo)[61]. The largest study to date included 148 patients with NELM who were treated with TARE and demonstrated a response rate of 70% with a median OS of 70 mo[66]. More recent studies include a retrospective study of 30 patients with NELM who were treated with TARE for a median OS for 39 mo[67]. In one retrospective study, 51 patients with NELM were treated with TARE and demonstrated 83% response by RECIST with median OS of 50.1 mo and PFS of 19.9 mo[14]. A randomized controlled pilot study of 11 patients compared TAE to TARE and found similar response rates by RECIST criteria at 6 mo [68]. A recent multi-institutional analysis found that both TACE and TARE were safe and effective liver-directed therapies for unresectable NELM. Although TACE demonstrated improved short-term disease control and response rates, both resulted in comparable long term outcomes[14].

Ablation in NELM

Ablation can be used alone or in conjunction with surgical resection. When used in conjunction with resection, it can both widen the candidates for resection and provide debulking in bilobar disease. Retrospective study of 16 patients who had a median of 23 liver metastases each were treated with resection and RFA and achieved a 3-year OS of 86 percent[69]. Another retrospective study of 40 patients treated with resection and RFA achieved PFS of 22 mo and median OS of 95 mo[70]. These findings are supported by a third retrospective study of 94 patients who underwent resection with intraoperative ablation, achieving a 5-year OS of 80% and 10-year OS of 59%[71]. Indeed, a recent population-based study found that 30% of patients undergoing resection of NELM also had concomitant ablation with no increase in perioperative morbidity[72]. In some cases, RFA may be as effective as surgical resection; a prospective study of 89 patients with NELM who were treated with RFA alone demonstrated symptom relief in 97% of patients, with a PFS of 16 mo and median OS of 72 mo. Further, the 5-year survival rate of 57% in this study is comparable to the 5-year survival rate of 61% seen in surgical resection[73]. MWA with or without concomitant resection has also been studied for NELM. In a phase II trial of 11 patients, complete ablation, defined as lack of enhancement on triple phase CT, was achieved in 90% of patients at 5 years[74].

Future directions

In addition to locoregional approaches, advances in the molecular understanding of neuroendocrine tumors has led to growing interest in the use of small molecule inhibitors for the treatment of neuroendocrine tumors. Sunitinib, a tyrosine kinase inhibitor, and everolimus, an mTOR inhibitor, have already been approved for use in neuroendocrine tumors[62,75]. An exciting new therapy for neuroendocrine tumors is peptide receptor radionucleotide therapy (PRRT), in which radionucleotides bound to SSA are delivered directly to somatostatin receptor positive tumors[76,77]. In fact, a phase 2 study of TARE with holmium-166 following PRRT is currently underway[78]. Additional research combining locoregional approaches with new therapeutics is needed to explore the benefits in the setting of liver-predominant disease.

BREAST CANCER

Introduction to breast cancer with liver metastasis

Breast cancer is a leading cause of mortality worldwide. Roughly 1 in 5 women with breast cancer will develop metastatic disease to the liver[12,79]. Breast cancer with liver metastasis (BCLM) typically occurs late in the disease course and is associated with a worse prognosis than metastasis to other sites like brain or bone. With treatment, median OS in BCLM is 14 mo[80]. For metastatic disease, systemic therapy remains the standard of care. For patients with isolated BCLM who respond to systemic chemotherapy, surgical resection can be offered[81]. However, recurrence rates even in highly selected patients remain high and the vast majority of patients harbor unresectable disease[12]. Given these limitations, local options like TAE, TACE, and TARE have been used for palliation and to enhance locoregional control.

TACE for BCLM

TACE is a palliative option for BCLM and has shown benefit as an adjunct to systemic

chemotherapy in retrospective studies. Li *et al*[82,83] compared DEB-TACE plus systemic chemotherapy with systemic chemotherapy alone in 47 patients, which demonstrated a median OS of 28 mo, the highest to date for TACE. This is consistent with more recent work by Duan *et al*[84] in 44 patients with liver-only metastatic disease, which demonstrated improved response rates (59.1% *vs* 34.9% by RECIST criteria) and improved survival at 1, 2, and 3 years. In the largest study to date, Vogl *et al*[85] demonstrated a median OS of 25-mo in 208 patients treated with c-TACE and systemic chemotherapy. More recently, a pilot study of DEB-TACE demonstrated disease control and median OS of 17 mo in 23 patients with chemo-resistant disease, though the treatment protocol was associated with adverse effects[86].

TARE for BCLM

TARE is an alternative palliative treatment with promising response rates in chemo resistant BCLM. A study of 81 patients with unresectable liver metastases demonstrated a median OS of 8 mo and a 61% response rate by PERCIST criteria[87]. Most recently, Deipolyi *et al*[88] demonstrated a response rate of 75% at 3-5 mo (PERCIST) and median OS of 15 mo. A recent review of 47 patients who received either TARE or TACE found that TARE was significantly better tolerated and demonstrated a trend toward improved survival. In this study, TARE was associated with a median OS of 13 mo and 3-month disease control in 47% of patients by mRECIST criteria[79].

Ablation for BCLM

For small isolated metastases, ablative therapy may be associated with similar survival outcomes with fewer adverse events compared to surgical resection[12,83]. Recent studies demonstrate median OS ranging from 30 to 70 mo[83]. One retrospective study of 69 patients with BCLM demonstrated PFS of 24 mo, with median OS of one-, two-, three- and five-year survival rates of 81.8, 50.1, 25.3 and 11.0%, respectively[89]. A more recent retrospective study of 33 patients with oligometastatic breast cancer demonstrated a median OS of 70 mo. Subgroup analysis of 14 patients with hepatic metastasis revealed PFS of 9 mo, which improved to 13 mo in patients who were able to achieve ablation of all metastatic disease in the liver[90]. Prognostic factors associated with improved tumor control and PFS across multiple trials include tumor size, estrogen receptor positivity, and 5-10 mm ablation margins.

Ablation is an appropriate therapy for tumor control of isolated liver metastases and reduces the need for time on systemic chemotherapy. Prospective randomized trials comparing systemic therapy alone to systemic therapy with ablation are needed to determine whether ablation offers a survival benefit as an adjunctive therapy. Further, given that ablation is associated with similar overall survival and decreased morbidity and mortality compared to resection, prospective randomized trials are needed to compare the two approaches to determine the appropriate standard of care.

Future directions

In addition to recent advances in locoregional strategies, new immunotherapies, pembrolizumab and atezolizumab, have been recently FDA-approved to treat metastatic breast cancer[91,92]. Additionally, the FDA recently approved the use of poly(ADP-ribose) polymerase (PARP) inhibitors olaparib and talazoparib in metastatic breast cancer[93,94]. There is growing interest in the synergistic effects of PARP inhibitors combined with radiotherapy, and additional studies are needed to determine outcomes in BCLM[95]. Future studies comparing combination strategies of immunotherapy, PARP inhibitors, and locoregional therapies like ablation and TARE are needed, as they may be able to demonstrate improved outcomes BCLM and strengthen the multidisciplinary care of BCLM.

LUNG CANCER

Introduction to lung cancer with liver metastasis

Lung cancer is the leading cause of cancer death worldwide and metastatic disease is associated with a 5-year survival rate of 4%. Liver metastasis in particular is associated with a worse prognosis compared to metastasis to the brain or bone and is most common in small cell lung carcinoma[96]. In this setting, treatment consists of palliative systemic chemotherapy. However, the 2018 TMN staging criteria distinguish between single and multiple extra thoracic metastasis, suggesting that a more aggressive approach to limited metastatic disease may improve outcomes. Data on

surgical resection is limited to a handful of case reports, which describe benefit in select patients[97-99].

Locoregional therapies for lung cancer with liver metastasis

The benefit of local therapies like TACE, TARE, and ablation are not well characterized for lung cancer with liver metastasis. Regarding TACE, review of a prospective multi-institutional registry containing 13 patients with liver metastasis who were treated with DEB-TACE using either doxorubicin or irinotecan revealed a response rate of 50% at 12 mo and a median OS of 14 mo[100]. Data on TARE is limited to nine patients discussed in two case reports and one retrospective review and demonstrate its potential as salvage therapy in chemo-refractory disease[101]. Regarding ablation, one retrospective review contained four patients with solitary liver metastasis from a primary non-small cell lung cancer who were treated with MWA. Treatment was well tolerated, but subgroup analysis on response rate and overall survival was not performed[102]. Despite the lack of prospective data, local therapies may provide benefit as adjunctive treatment in select patients with oligometastatic disease.

UVEAL MELANOMA

Introduction to uveal melanoma with liver metastasis

Uveal melanoma is the most common primary malignant intraocular tumor in adults. Nearly half of patients will develop metastatic disease, and of those, over 90% will have primary metastasis to the liver. While surgical resection remains the standard of care when feasible, less than 10% of patients will be candidates for surgical resection [10]. Additionally, metastatic uveal melanoma is generally unresponsive to systemic chemotherapy. Without treatment, prognosis for metastatic disease is poor, with median survival of less than nine months[10]. As no medical therapies have yet been shown to prolong survival, there are currently no FDA-approved therapies for metastatic disease. Notably, the major advances in metastatic cutaneous melanoma using immunotherapy have not yet been replicated in metastatic uveal melanoma. Therapeutic approaches under investigation include systemic immunotherapy with checkpoint inhibitors nivolumab and ipilimumab as well as a number of locoregional approaches including ablation, radio- and chemoembolization, and intrahepatic perfusion. The rarity of metastatic uveal melanoma presents a challenge for study design, and many studies comprise cohorts of 20 to 50 patients with wide ranges in outcome measurements between studies.

Locoregional therapies for uveal melanoma with liver metastasis

Recent work by Höppener *et al*[103] provides a meta-analysis of locoregional approaches in uveal melanoma with metastasis to the liver. The vast majority of studies are retrospective cohort studies and outcomes examined include tumor control, progression free survival, and overall survival. In 19 studies of TACE median OS was 6 mo (range 5 to 28). Cisplatin was the most common chemotherapeutic used, and others included doxorubicin, mitomycin-c, fotemustine, and irinotecan. Of these, two studies compared TACE to systemic chemotherapy and found no difference in overall survival[104,105]. Thirteen studies of SIRT with ^{90}Y demonstrated a median OS of 11 mo (range 4-26) and included a recent phase II trial showing median survival of 10 mo [10]. Six studies of ablation (predominantly RFA) demonstrated median OS of 19 mo (range: 11-46) and included a recent phase Ib/II trial that combined RFA with ipilimumab to little clinical effect[106]. Fourteen studies examined intrahepatic perfusion of melphalan, a unique approach which involves the introduction of melphalan *via* the hepatic arteries paired with IVC bypass with melphalan filtration to prevent systemic circulation of chemotherapy. This approach demonstrated a median OS of 11 mo (range 5-27). While comparable in terms of overall survival, unique adverse events have been reported using this approach due to the cardiovascular and coagulopathic risks of bypass.

Future directions

Given the lack of a standard of care for metastatic uveal melanoma, many other approaches are being developed alongside locoregional therapies. In addition to the locoregional approaches above, hepatic artery infusion with fotemustine has shown potential benefit in uveal melanoma with liver metastasis[107]. Seventeen studies of hepatic artery infusion with fotemustine had a median OS of 13 mo (range 3-21). In the

last ten years, the development of immune checkpoint inhibitor therapies including ipilimumab, nivolumab and pembrolizumab have transformed outcomes for malignant melanoma[108]. While these results have not yet been replicated in uveal melanoma, trials of combination therapy have shown some potential benefit[109]. Other experimental agents for uveal metastatic melanoma include tumor infiltrating lymphocytes[110], epigenetic therapies[111-113], and tebentafusp, a bispecific fusion protein that targets CD3+ and T-cell receptors[114]. Additional research is needed regarding the application of these therapies in a neoadjuvant or adjuvant setting to improve the multidisciplinary care of metastatic uveal melanoma.

CHOLANGIOCARCINOMA

Introduction to intrahepatic cholangiocarcinoma

Cholangiocarcinoma is a rare malignancy of the biliary system that is occurring with increasing incidence and can be anatomically divided into intrahepatic, perihilar, and extrahepatic types. Given the aggressive and asymptomatic course of early disease, late presentation is common. While the current standard of therapy is resection, recurrence is seen in 60% of patients[115,116]. Further, about 75% of patients are not candidates for resection at time of presentation due to tumor size, location, multifocality, or distant metastatic disease[117]. In the case of unresectable disease, the current standard of care is systemic platinum-based chemotherapy plus gemcitabine, which confers a median OS 11.7 mo based on the results of the ABC-02 trial[118]. In the decade since this trial, studies have shown benefits associated with locoregional therapies in the treatment of intrahepatic cholangiocarcinoma (IHC), including improved overall survival and successful downstaging to surgical intervention[119].

TACE in IHC

With regard to trans-arterial chemoembolization, both c-TACE and DEB-TACE have been investigated in patients with IHC. Park *et al*[120] demonstrated improved OS in patients treated with c-TACE *vs* supportive care, and meta-analysis of 542 patients with IHC treated with c-TACE reveals a median OS of 13.4 mo after treatment[121]. In a study of 24 patients, DEB-TACE was associated with median OS of 17.5 mo[122], and studies of combination DEB-TACE with systemic chemotherapy have demonstrated higher median overall survival than treatment with chemotherapy alone[123,124]. In a three-way comparison of systemic chemotherapy, c-TACE, and DEB-TACE in patients with IHC, DEB-TACE was associated with greater OS than c-TACE, and similar OS to systemic chemotherapy[125]. The ongoing CTILC study (NCT03317483) investigating DEB-TACE in various liver cancers includes 37 patients with IHC[126].

TARE in IHC

Radioembolization with ^{90}Y plus first-line chemotherapy has been shown to increase overall survival and successfully downstage patients to surgical resection. A recent single-center retrospective study of 85 patients showed median OS of 21 mo from time of diagnosis and median OS of 12 mo after treatment[127]. These findings were further supported by a multicenter retrospective study of 115 patients showing median OS from diagnosis of 29 mo with median OS after treatment of 11 mo[128]. Within the last year, Edeline *et al*[129] published the results of a phase 2 trial of ^{90}Y with first-line chemotherapy in 41 patients with a response rate of 40%, median OS of 22 mo, and successful downstaging of over 20% of trial participants. Given the significant improvement in overall survival compared to the current standard of care, a phase 3 trial is ongoing[129].

Ablation in IHC

Ablation is an option in select patients who are poor surgical candidates and have early-stage IHC (< 5 cm)[130]. Given these criteria, few patients are candidates and available study sizes are small. Recent metanalysis of 10 studies with a total of 206 patients showed median OS for patients treated with RFA ranged from 8.7 to 52.4 mo [131]. Preliminary research suggests that MWA confer a survival benefit *vs* RFA in tumors less than 3cm[132]. Given the invasive nature of the disease, multiple authors recommend wide ablation margins[133-135]. No studies have specifically investigated the role of cryoablation or irreversible electroporation for IHC[136].

Future directions in IHC

Given the importance of chemotherapy in the treatment of IHC, ongoing trials of DEB-TACE represent an exciting area of research. Additionally, the development of targeted therapies for IHC is an area of active research and may eventually be used in conjunction with locoregional approaches to improve outcomes. Currently, phase III trials of the isocitrate dehydrogenase 1 (IDH-1) inhibitor ivosidenib and fibroblast growth factor receptor (FGFR) inhibitors are ongoing[117]. Finally, there are promising results from studies of hepatic artery infusion with floxuridine (FUDR-HAI) combined with first-line chemotherapy[137-140]. While the role of interventional liver-directed therapies continues to expand, it remains to be seen how new targeted approaches can be combined with locoregional strategies to improve multidisciplinary care of IHC.

SARCOMA

Introduction to sarcoma with liver metastasis

Sarcomas are a diverse set of tumors that arise from mesenchymal cells in various parts of the body. These mesenchymal cells can differentiate into a variety of tissues including muscle, adipose, cartilage, nerve, and vascular tissue. Prognosis is related to tumor type — gastrointestinal stromal tumors, for example, are associated with better prognosis, while leiomyosarcomas, which are notoriously resistant to systemic chemotherapies are associated with poor prognosis[141]. In all types, the feared complication is hematogenous metastasis, which is considered incurable and associated with median survival of 12 to 19 mo. In metastatic disease, palliative chemotherapy is the standard of care, despite the fact that only 10-25% of metastatic sarcomas respond to systemic chemotherapy[142]. There is growing interest in more aggressive local treatment, especially for oligometastatic disease. While complete surgical resection is preferred, many patients are not surgical candidates. In unresectable, recurrent or chemo-resistant disease, local therapies like TAE, TACE, and ablation are associated with increased tumor response and overall survival.

TAE in sarcoma with liver metastasis

Two studies of TAE demonstrated improved response rate and overall survival in patients with unresectable, chemoresistant sarcoma with liver metastasis (SLM). The first was a retrospective study of patients with hepatic metastasis that was either incompletely resectable or had failed other therapies. Treatment response was defined as greater than 25% reduction in tumor size or greater than 50% necrosis and achieved response in 9 of 15 patients. OS was 62%, 41%, and 29% at 1-, 2-, and 3 years respectively[143]. The second study examined TAE in 11 patients with GIST that had metastasized to the liver in patients who had either been treated with first line imatinib alone or first-line imatinib followed by and second line sunitinib. In the first group, median survival was 15 mo and PFS was 3.8 mo. In the second group, TAE achieved a median OS of 24 mo and PFS of 3.4 mo. Response rate was 46% overall by mRECIST criteria[144]. GIST tumor type and radiographic response were both associated with prolonged survival. These results represent improvement in both response rate and overall survival compared to treatment with second- or third-line chemotherapy.

TACE in SLM

Three retrospective studies examine the use of TACE in sarcomas with liver metastasis. The earliest, in 1995, used cisplatin beads with vinblastine arterial infusion in 14 patients with gastrointestinal leiomyosarcoma with prior resection. However, local therapy with cisplatin and vinblastine induced > 50% reduction in tumor size in 70% of patients with median PFS of 12 mo[145]. These findings are supported by a retrospective review of 16 patients, most with leiomyosarcoma, which demonstrated tumor control or response in 83% of patients and a median OS of 20 mo after treatment with cisplatin, doxorubicin, and mitomycin-C[146]. Most recently, a retrospective study of 30 patients treated with c-TACE using doxorubicin, cisplatin, and mitomycin-C demonstrated a response of 48% by mRECIST criteria, PFS of 6.3 mo, and median OS of 21 mo[142]. These studies reveal that TACE is an appealing option, particularly in the treatment of leiomyosarcomas, which are highly resistant to systemic chemotherapy.

Ablation in SLM

Ablation for SLM has been examined in three retrospective studies. The first included 66 patients with SLM who were treated with either surgical resection, RFA, or combination therapy. Of the 18 patients who underwent surgical resection with RFA and 13 patients who underwent RFA alone, PFS was 7.4 mo and median OS was 33.2 mo [147]. The second retrospective study comprised 13 patients with GIST with liver metastasis and 12 patients with other sarcoma subtypes with liver metastasis. Of the patients with GIST, 85% showed tumor response with a single treatment of RFA, and non-responders were treated with a second round of RFA, achieving total response. Patients with GIST demonstrated PFS of 28 mo. In other tumor types with liver metastasis, response was observed in 71% of patients, with PFS of 7 mo[148]. Most recently, data from a large retrospective study of 281 patients with metastatic sarcoma support the use of RFA in non-resectable metastatic disease[149]. In addition to these retrospective studies, there are a number of recent case reports on RFA in SLM[150-152]. Ablation is generally well-tolerated and is associated with greatly improved tumor response, progression free, and overall survival in patients, particularly in patients with unresectable or chemo resistant SLM. Further, RFA and surgery can be used in conjunction in many sarcoma subtypes to maximize outcomes.

Future directions

Given the diversity of sarcomas, there is ongoing research into a number of small molecule inhibitors for specific sarcoma subtypes[153]. For metastatic sarcoma in general, preliminary research demonstrates promising outcomes with tivozanib, a VEGF inhibitor[154], and a new chemotherapeutic, eribulin, which has demonstrated benefit in combination with dacarbazine[155]. Additional studies exploring the use of these therapies in conjunction with DEB-TACE would elucidate the role for these therapies in liver-predominant disease.

CONCLUSION

Metastatic disease to the liver is the most common malignant liver condition and a major cause of cancer-related morbidity and mortality. Surgical resection and systemic chemotherapy remain the standard of care in most types of metastatic liver disease, but there is an expanding role for locoregional therapies in liver metastasis with various aims including curative intent, tumor control, downstaging to resection, symptom control, and palliation. TAE, which can be combined with chemotherapy and/or radiotherapy, has the potential to improve tumor response rates and disease-free and overall survival in select patients. Ablative procedures using high frequency alternating currents or microwaves represent comparable alternatives to resection and can even achieve curative results in selected patients. Combined with advances in immunotherapy and targeted therapies, advances in locoregional approaches are providing more robust, multidisciplinary treatment options for metastatic liver disease.

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REVIEW

Hematopoietic stem cell mobilization strategies to support high-dose chemotherapy: A focus on relapsed/refractory germ cell tumors

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Abstract

High-dose chemotherapy (HDCT) with autologous hematopoietic stem cell transplantation has been explored and has played an important role in the management of patients with high-risk germ cell tumors (GCTs) who failed to be cured by conventional chemotherapy. Hematopoietic stem cells (HSCs) collected from the peripheral blood, after appropriate pharmacologic mobilization, have largely replaced bone marrow as the principal source of HSCs in transplants. As it is currently common practice to perform tandem or multiple sequential cycles of HDCT, it is anticipated that collection of large numbers of HSCs from the peripheral blood is a prerequisite for the success of the procedure. Moreover, the CD34+ cell dose/kg of body weight infused after HDCT has proven to be a major determinant of hematopoietic engraftment, with patients who receive > 2×10^6 CD34+ cells/kg having consistent, rapid, and sustained hematopoietic recovery. However, many patients with relapsed/refractory GCTs have been exposed to multiple cycles of myelosuppressive chemotherapy, which compromises the efficacy of HSC mobilization with granulocyte colony-stimulating factor with or without chemotherapy. Therefore, alternative strategies that use novel agents in combination with traditional mobilizing regimens are required. Herein, after an overview of the mechanisms of HSCs mobilization, we review the existing literature regarding studies reporting various HSC mobilization approaches in patients with relapsed/refractory GCTs, and finally report newer experimental mobilization strategies employing novel agents that have been applied in other hematologic or solid malignancies.

Key Words: Hematopoietic stem cells; Germ cell tumors; Hematopoietic stem cell transplantation; Granulocyte colony-stimulating factor; Plerixafor

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Core tip: High-dose chemotherapy (HDCT) followed by autologous stem cell transplantation (ASCT) is a curative treatment option for patients with relapsed/refractory germ cell tumors (GCTs). Mobilization of adequate numbers of hematopoietic stem cells (HSCs) is a prerequisite for successful ASCT. As the benefit of HDCT+ASCT is largely evident with > one HDCT cycle, it is anticipated that an appreciable percentage of patients will not mobilize adequate HSCs and require salvage strategies. Herein, we review the history of HSC transplantation, with emphasis in GCTs, pathophysiological mechanisms of HSC mobilization, initial and salvage mobilization strategies, and finally discuss novel mobilizing agents and approaches to overcome failures.

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INTRODUCTION

High-dose chemotherapy (HDCT) followed by autologous hematopoietic stem cell transplantation (ASCT) has been a major breakthrough in oncology. It has broad applicability in patients with metastatic germ cell tumors (GCTs) who experience one or even more relapses after previous chemotherapy, or in those with a poor prognosis on diagnosis (*e.g.*, with extragonadal primary or incomplete response to first-line cisplatin-based chemotherapy)[1,2]. The efficacy of HDCT and ASCT depends largely on successful and adequate hematopoietic stem cell (HSC) mobilization, which ensures faster neutrophil and platelet engraftment and therefore decreased infection risk and hospitalization[2]. Collection of at least 2.0 × 10^6 CD34+ HSCs has been considered the minimum for a subsequent successful ASCT[3,4]. However, successful mobilization remains a great challenge, as a significant number of patients, somewhere between 5%-30%, are unable to mobilize enough HSCs to support subsequent ASCT. That has been attributed to extensive and prolonged prior exposure to bone marrow-suppressing intensive chemotherapy that has ultimately led to poor bone marrow reserves [5]. Indications, as far as strategies appropriate for achieving adequate CD34+ cell numbers for these patients, are limited by a lack of data and are generally based on standard approaches for HSC mobilization that have been applied in other disease settings. Hence, the establishment of standard mobilization and remobilization techniques for patients with GCTs who failed the initial mobilization protocols should become a high priority (outlined in Figure 1).

GERM CELL TUMORS

Testicular cancer and GCTs typically subdivided into two main histologic subtypes, seminomas and non-seminomas, are the most common solid tumor in men between 20 and 35 years of age[6,7]. Approximately 50% of testicular cancers are non-seminomas, which are typically more malignant and usually associated with a more aggressive clinical presentation[8]. The cure rates are between 41%-92%[9,10]. About 20%-30% of patients with metastatic disease at initial presentation will eventually require salvage treatment. Second-line therapy options include conventional dose cisplatin-based regimens, or high-dose chemotherapy regimens, currently consisting of carboplatin and etoposide plus ASCT support[10,11].

To date, the main conventional dose chemotherapy (CDCT) salvage regimens include etoposide-ifosfamide-cisplatin, vinblastine-ifosfamide-cisplatin, and paclitaxel (taxol)-ifosfamide-cisplatin (TIP)[12,13]. Randomized data are lacking, and retrospective comparisons have failed to demonstrate the superiority of any of these regimens. Nevertheless, the best results were observed with TIP, which is therefore currently broadly accepted as the optimal choice of salvage chemotherapy.

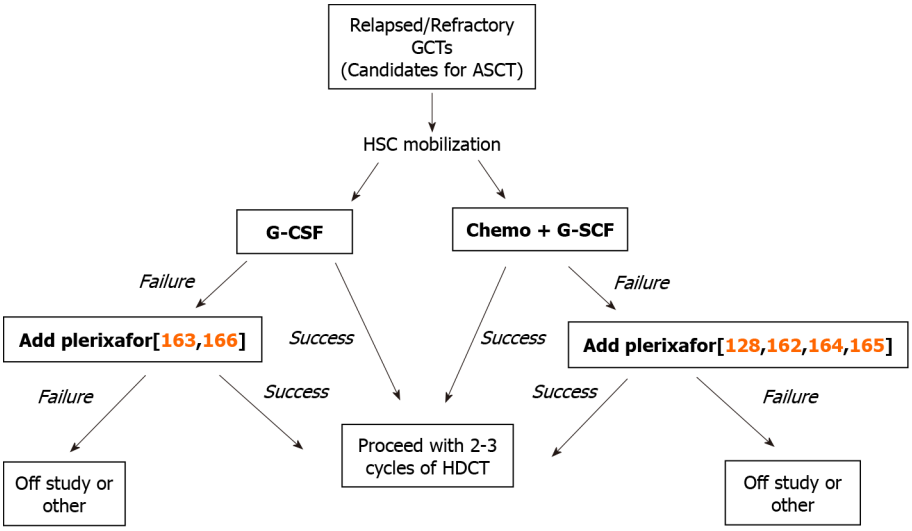


Figure 1 Mobilization algorithms. ASCT: Autologous stem cell transplantation; G-CSF: Granulocyte colony-stimulating factor; GCTs: Germ cell tumors; HDCT: High-dose chemotherapy; HSC: Hematopoietic stem cell.

CURRENT STATUS OF HDCT AND ASCT IN GERM CELL TUMORS

In HDCT, cytotoxic agents are administered at much higher doses than the standard dose applied in CDCT. The observation of a larger therapeutic impact even at minor increases of dosage, proved the dose-response relationship of many chemotherapeutic agents, and thus supported the efficiency of HDCT regimens in eradicating residual drug-resistant tumor cells[14]. Increased doses lead also to more severe side effects, with prolonged myelosuppression being the main reason to delay subsequent cycles, thus leading to failure[15]. To reduce the duration of pancytopenia, and therefore the failure rate, HSCs are harvested from the patient's peripheral blood by apheresis before the administration of HDCT. After completion of HDCT the harvested stem cells are reinfused to repopulate the bone marrow and ultimately re-establish hematopoiesis. Despite the fact that the use of HDCT as salvage in GCTs is a standard treatment option for most patients, its efficacy as a first salvage strategy remains a matter of debate among investigators[16-19]. An ongoing phase III trial - the TIGER study - may be the first to establish HDCT as initial salvage in these patients, considering the existing inconsistent evidence as well as the lack of conclusive randomized trials.

HISTORY OF ASCT

Total-body irradiation (TBI) prior to autologous transplantation was first applied in animals in the 1930's. The early studies had fatal outcomes because of severe gastrointestinal and nervous system complications, hemorrhage, and infection[1,2]. Similar trials of TBI were performed in humans few years later. The first was performed by Thomas and his colleagues in a leukemic patient, who was grafted with bone marrow from her identical twin sister. They reported a 3-month remission duration in this patient. Following the discovery of the human leucocyte antigen (HLA) system by Dausset in 1958[20], the concept of histocompatibility, *i.e.* identical HLA in both the donor and recipient (patient),was applied, with high success rates for allogeneic transplantations.

STEM CELL SOURCES-DIFFERENCES BETWEEN PERIPHERAL BLOOD HSCs AND BONE MARROW HARVESTING

Bone marrow was the first source of HSCs, which were obtained by repeated aspirations from the posterior iliac crests with the donor under general or local anesthesia. The method was used for many years until the observation that stem cells detach, enter the circulation and home to the marrow. After that observation, peripheral blood

harvesting, as more convenient and appropriate source of HSC, has replaced bone marrow[1]. There are two types of peripheral blood leukapheresis, normal volume and large volume. The normal volume procedure processes 2.5 to 3 times the patient blood volume. The large volume procedure processes 4-5 times the volume. Many researchers evaluated the efficacy and safety of large volume leukapheresis and concluded that, after successful mobilization, this leads to a higher CD34+ cell harvest without a change in graft quality ,with fewer sessions to reach greater than 2 × 10^6 CD34+ cells/kg body weight[3,4,21].

Goldman *et al*[22] was the first to use HSCs collected from the peripheral blood for autologous transplantation after high-dose cytotoxic therapy in patients with CML. Körbling *et al*[23] followed with a report of autologous transplantation in a patient with CML, and a patient with Burkitt's lymphoma. Körbling *et al*[23] reported the collection of peripheral blood stem cells after the use of granulocyte-macrophage colony-stimulating factor (GM-CSF) during leukocyte recovery after myelosuppressive chemotherapy. That was the first example of chemotherapy-induced "mobilization". Subsequently Kessinger *et al*[24] used the same mobilization method and documented that performing multiple leukapheresis sessions resulted in a sufficient number of circulating HSCs in the peripheral blood to ensure engraftment after HDCT.

DIFFERENCES BETWEEN PERIPHERAL BLOOD HSC AND BONE MARROW HARVESTING

Traditionally, as HSCs reside in the bone marrow at steady-state conditions, collection has been carried out by bone marrow harvesting from the posterior iliac crests and possibly the sternum under general or epidural anesthesia[25]. Bone marrow harvesting, as mentioned earlier, is a one-time procedure with multiple risks that increase with donors age and comorbidities. Peripheral blood HSC (PBSC) collection performed by large-volume leukapheresis, is dependent on stem cell mobilization, and a prolonged harvesting period is required. However it is considered safe to perform on donors without the need of any type of anesthesia. A limitation of PBSC collection is adequate venous access. PBSC collection performed by single or multiple apheresis avoids the risks of general anesthesia and shortens the time for hematopoietic recovery. The most common adverse effects include moderate-to-severe bone pain as a result of leucocyte growth factor administration, fatigue, and headache. Rare adverse events include splenic rupture, acute arthritis, anaphylaxis, and cardiac ischemia[26-28].

Since the early 90's, HSCs mobilized from the bone marrow into the peripheral blood (PB) have been established as the preferred source of HSCs for transplantation because they are easily accessible, and the evidence indicates that they engraft faster after transplantation than HSCs directly harvested from bone marrow (BM). Clinical findings from randomized/comparative trials indicate that patients experience faster neutrophil, platelet, and immune recovery after PB stem cell transplantation; and in allogeneic transplantation, a higher incidence of chronic graft *vs* host disease and lower probability of relapse[29].

HSCS MOBILIZING AGENTS

HSCs are multipotent precursors with self-renewal potency that reside predominantly in the bone marrow. A small number of HSCs circulate in the blood (< 0.02%) under steady-state conditions[30]. Several methods have demonstrated effectiveness in increasing the percentage of HSCs in PB and maximize the number collected with the intention of restoring marrow function and reduce the time required for neutrophil and platelet engraftment following HDCT. Initial mobilization strategies include: (1) Administration of hematopoietic CSFs alone; (2) A course of myelosuppressive chemotherapy prior to collection; and (3) Chemotherapy followed by cytokine administration. Remobilization strategies include: (1) Dose escalation of leucocyte CSFs; granulocyte (G)-CSF or granulocyte-macrophage (GM)-CSF, with or without IL-3; (2) Different forms of G-CSF, with altered glycosylation patterns to improve pharmacokinetics and bioavailability; (3) G-CSF in combination with other HSC mobilizing agents, *i.e.* Plerixafor or stem cell factor (SCF), *kit*-ligand (known as ancestim); and (4) G-CSF in combination with chemotherapy and newer agents like plerixafor. A course of myelosuppressive chemotherapy prior to HDCT as a chemo-mobilization strategy

not only increases stem cell collection, but also provides better control of the underlying malignancy, when active agents or chemotherapy regimens are administered [31,32]. However, an increased risk of infection and hospitalization is expected in patients undergoing chemo-mobilization[31].

In turn, the administration of mobilization agents alone not only has the benefit of relatively predictable kinetics of mobilization, but also a reduced need for hospital care compared with chemotherapy because of the minimal side effects of G-CSF[33,34]. The most commonly used myeloid growth factor for peripheral stem cell harvesting is G-CSF. Other alternatives are its pegylated form; pegfilgrastim, and sargramostim; the recombinant human GM-CSF. Several studies now confirm higher successful rates and twice as many progenitor cells in the circulation when a combination of chemotherapy and G-CSF is used. Consequently, that approach is favored by many investigators[35, 36].

Having said that, the use of newer agents, such as chemokine receptor antagonists, along with the conventional ways of autografting mentioned above has expanded in recent years, with promising synergistic results. Plerixafor, a bicyclam molecule derivative that reversibly competes with and inhibits stromal-derived factor-1a (SDF-1a; also known as CXCL12) binding to CXCR4, causes an absolute peak of CD34+ cells 6-9 h after administration. Administration is preferable in the evening before apheresis, ideally 8-10 h before the procedure to maximize the number of HSCs collected [37]. Daily administration of plerixafor in the evening for up to four consecutive days can be given, with a morning G-CSF dose along with the apheresis sessions if the desired HSC target number has not been achieved[38]. However, considering the higher cost of that approach, one recognizes the need to establish specific mobilization algorithms in order to maximize the potential of the conventional mobilization agents. That improves the pharmaco-economics of mobilization and reduces the need of rescue remobilization with plerixafor. Nowadays, because of its high cost, plerixafor use is restricted to patients failing to reach sufficient PB CD34+ cell counts (*i.e.* preemptive application) on the day that apheresis is planned to start or in patients failing to collect sufficient CD34+ cells during leukapheresis (*i.e.* rescue application). Preemptive use of plerixafor, especially in combination with G-CSF in poor mobilizers has proven to be more cost effective[39,40].

MOBILIZATION ALGORITHMS TO OPTIMIZE MOBILIZATION OUTCOMES

In patients with relapsed/refractory GCTs, we and others attempt HSC mobilization preferably after 1 or 2 salvage chemotherapy cycles with TIP or TI followed by the administration of G-CSF between days 3 and 11 or until the day when sufficient numbers of CD34+ HSCs have been obtained. This approach is accompanied by frequent measurement of circulating PB CD34+/μL counts by flow cytometry, usually starting on day 10-11, in order to decide when to perform the apheresis. A mobilization algorithm called the "just in time"[41] approach helps to decide whether the patient is in need of plerixafor. Patients with an absolute number of CD34+ cells > 3 and < 15/μL are the main candidates for plerixafor administration. Other protocols include "one size fits all"[42], in which a standard technique is applicable to all patients and "risk-based approaches"[43]. The latter places patients into categories, where those who meet more of the predefined criteria are more likely to be poor mobilizers, and thus a different approach must be used. Poor mobilizers are defined as those who have received many prior lines and cycles of chemotherapy, particularly those who have been exposed to alkylating agents, irradiation, pre-existing low blood counts, bone marrow involvement by the tumor, and advanced age[39,44].

UNDERSTANDING THE STEM CELL NICHE IS CRITICAL FOR FURTHER PHARMACOLOGICAL STUDIES

Schofield was the first to propose the concept of HSCs in 1978[45]. Since then, many have attempted to virtually define this area[46-49], and as a result, we now refer to stem cell niche as the microenvironment where localization and regulation of stem cells takes place. The area is anatomically located near to the endosteum and is composed by two major compartments, the perivascular and the endosteal niches, where cells and molecules dynamically interact[50,51]. The endosteal niche compartment consists of osteoblasts and is critical for supporting the lymphoid progenitors

[52]. It is a hypoxic environment that favors the undifferentiated state of HSCs[53], where low energy supplies are needed. Hypoxia is a critical component of the HSC niche[54], and exposure of HSCs to elevated oxygen tissues negatively affects self-renewal and promotes cell cycle entry, hindering low-cycling proliferation[54,55]. Low oxygen concentration in the endosteal niche is regulated by hypoxia-inducible factor-1 (HIF-1), a transcription factor, which under hypoxic conditions, binds in its full heterodimeric form (HIF1a + HIF1b) to DNA elements controlling transcription of various genes related to angiogenesis and erythropoiesis, resulting in the upregulation of vascular-endothelial growth (VEGF), which ultimately leads to vasodilation and HSC mobilization[56].

The vascular niche is rich in oxygen, and it is thought that HSCs migrating towards the niche proliferate and regenerate. This compartment is subcategorized into arterial-perivascular, mesenchymal, and sinusoidal endothelial niches. Recent studies showed that the arterial-perivascular niche mostly consists of nestin-bright (nestin+)-smooth muscle perivascular cells[57,58] that express high levels of CXCL12/SDF1 under steady-state conditions and therefore appear to be strongly associated with both proliferation and maintenance of primitive hematopoietic cells in a quiescent state[58,59]. The endothelial sinusoidal niche is composed of endothelial cells that are nestin-dim/leptin receptor-2 (LEPR2) and CXCL12-abudant reticular (CAR) cells with high amounts of CXC-L12, which contribute to regeneration after myelotoxic stress[58]. Several studies showed that as HSCs enter the cell cycle they relocate from areas rich in nestin-bright perivascular cells to those rich in LEPR2+ cells and are mobilized into the circulation[58-60]. In addition to cellular interactions, stem cells are attracted to the bone marrow niche cells through dynamic interactions involving soluble factors (*e.g.*, growth factors, chemokines and cytokines, and adhesion molecules).

One of the most critical chemotactic factors, SDF1a (CXCL12), mainly derived from osteoblasts and endothelial cells, attract HSCs by attaching to their surface chemokine receptor; CXCR4[61]. Other important adhesion molecules are VCAM1 (CD106), which binds to integrin α4β1, very late antigen-4 (VLA-4) on HSCs, and a transmembrane SCF that binds to c-kit (CD117) on HSCs[62,63]. It is well understood that the breaking down of those tethers is necessary for the release of HSCs into the circulation.

Other cells, such as adipocytes, and macrophages have supporting roles in the BM environment. CD169 macrophages secrete oncostatin-M, which leads to increased CXCL12 production by nestin+ and other mesenchymal cells *via* the MAPK-p38 signaling pathway[64,65]. Depletion of the macrophages results in downregulation of VCAM1, SDF1a, and SCF expression that disrupts the normal niche functions[64,65]. The percentage of adipocytes in the BM, derived from mesenchymal cells, increases with age, leading to a fatty marrow with limited cell proliferation ability[66].

INITIAL MOBILIZATION STRATEGIES

*Use of G-CSF or biosimilar**

Brief history: In 1966, Ray Bradley and Don Metcalf were the first to identify agents that can stimulate colony formation in hematopoietic cells in semi-solid culture[67]. Later, in 1985 Welte *et al*[68] purified human G-CSF. Nagata *et al*[69] in Japan and independently Souza *et al*[70] from AMGEN in 1986 cloned the G-CSF gene, resulting in the production and clinical application of this cytokine. The first preclinical data to demonstrate mobilization of hematopoietic cells following the administration of G-CSF in mice was in 1986 in a study conducted by Tamura *et al*[71], where an observation of increasing neutrophil counts approximately 2 h after injection made. The following year, Duhrsen *et al*[72], confirmed the mobilizing activity of G-CSF in cancer patients, where an increase of mature and progenitor cells into the circulation was observed. The observations were the stimuli for further animal studies to determine whether the progenitor cells could be effective for hematopoietic reconstitution[73].

Mechanism of action: The G-CSF receptor (G-CSFR) is expressed on a range of hematopoietic cells, including mature neutrophilic granulocytes, myeloid progenitors, and HSCs[74]. After binding to its ligand, receptor multimerization and activation of several intracellular signaling cascades occur, including the Jak/Stat/Socs, Ras/Raf/Erk and PI3-kinase/Akt pathways, which ultimately leads to transcriptional changes that have an impact on survival, migration, proliferation, and differentiation[74]. G-CSFR signaling also mediates the mobilization of hematopoietic progenitor cells (HPCs) and mature neutrophilic granulocytes from the bone marrow[75]. Multiple mechanisms have been described to explain the mechanism of action of G-CSF.

Because most of the topics are still poorly understood, further studies are required. It has been previously hypothesized that the mechanism of mobilization by G-CSF is indirect, based on the fact that HSCs themselves, in order to mobilize, do not express the G-CSFR receptor[76], which is mainly expressed on the surface of macrophages and osteomacs[77]. (1) The first mechanism includes the role of proteases. It is known that following G-CSF administration, an increase in the number of granulocytes occurs. The increase is accompanied by the production of large amounts of proteases such as neutrophil elastase, cathepsin, and MMP-9 by neutrophils[78], which in combination with other proteases, such as the CD26 dipeptidase[79], inactivate multiple adhesion molecules (VCAM1, CXCR4, fibronectin, c-kit, SCF, OPN), thereby disrupting their attachment to the VLA4 receptor and weakening intracellular adhesive interactions[80-83]. One of the most important mechanism is the induced proteolytic clearance and degradation of SDF1 (CXCL12) in the bone marrow. Matrix metalloproteinase (MMP)-9[84,85] and CD26 cause the cleavage of the NH2-terminal of SDF1, so it can no longer contact the surface CXCR4 receptor, leading to liberation of HSCs into the circulation[80,86]. In addition, type 1 metalloproteinase (MMP1) increases CD44 cleavage. CD44 ligand is hyaluronic acid, rich in endosteum and sinusoidal endothelium, and essential for HSCs homing[87]. (2) The second involves changes in bone formation. Following G-CSF administration, a variety of changes in bone formation occur, more specifically an almost complete loss of the osteoblastic layer has been observed[65,75,88]. Osteoblasts are essential in the BM microenvironment by producing cytokines, chemokines and adhesion molecules[89]. The osteoblasts, however, do not express the G-CSFR[88,90], which suggests that this effect is mediated by other cell types. Osteoclasts arise from HSCs and do express the G-CSF receptor, so it has been proposed that they play a critical role not only in formation of the hematopoietic niche, but also in HSC mobilization through secretion of cathepsin K, which cleaves and inactivates CXCL12[76,91]. However, the formation is no longer thought to be mainly the result of osteoclast activation, but rather to the loss of supporting cells, such as osteomacs and macrophages[65]. There is evidence that after administration of G-CSF, osteomacs leave the endosteal surface concurrent with endosteal osteoblast depletion[65]. (3) The third assumes a role of CD68/CD169 macrophages. The depletion of CD68/CD169+ macrophages seems to initiate a decreased expression of factors required for HSC retention (CXCL12), by selective downregulation of nestin+ mesenchymal stem cells (MSCs), as has been mentioned earlier[64,65]. That ultimately causes mobilization of HSCs into the PB. (4) The fourth involves complement activation. Activation of the complement cascade and thrombolytic pathway plays also a major role because of the release of sphingosine-1-phosphate (S1P) into the circulation by red blood cells, endothelial cells, and activated platelets. S1P is a strong chemoattractant of HSCs, creating an enabling environment for proliferation in the plasma[92,93]. S1P increases in blood and decreases in BM during mobilization, inhibiting SDF1 through the p38/Akt/mTOR pathway[92]. Both SDF1 and S1P are regulated by specificity protein (SP)-1, which it is thought to maintain a balance of their antagonistic effects. Several studies also suggest a role of the C5a complement component in mobilization, probably by neutrophil stimulation and the subsequent increase of MMP9 and decrease of CXCR4 expression. That is supported by the observation that C5-deficient mice respond poorly to G-CSF mobilization[94]. On the other hand, C3a expression promotes the chemotaxis of HSCs by CXCL12[94]. And (5) The fifth includes a role of the sympathetic nervous system. The role of the sympathetic nervous system (SNS) in G-CSF mobilization has been investigated. Sympathectomy or pharmacological innervation of the SNS[90] both lead to impaired mobilization in the mouse, and beta-2 (β2) agonist administration increases mobilization[90]. Another possible explanation is mobilization *via* nestin+ MSCs, which express many adhesion molecules, such as CXCL12, IL-17, and VCAM that are downregulated by β3 adrenoreceptor activation or G-CSF stimulation[95,96]. That observation explains why diabetes patients with impaired SNS function fail to mobilize adequate HSC numbers[97,98]. Summarizing, G-CSF upregulates CXCR4 in HPCs and decreases CXCL12 levels in the bone marrow relative to the blood and other tissues, establishing a chemo-attractive gradient that promotes migration of HSCs to the peripheral circulation.

Addition of chemotherapy as a mobilization strategy

For years there have been trials to establish a universal chemotherapeutic regimen, but without success because of uncontrolled or unknown variables. The optimal chemotherapeutic regimen for mobilization should have both antitumor activity and mobilization capacity[99]. Therefore, a chemotherapy regimen that is effective for the

underlying disease, either at relapse or first-line, in combination with G-CSF is used for PBSC mobilization. The main disadvantages are hematological toxicities, mobilization costs, and a rather unpredictable post-chemotherapy time for HSC harvest. Furthermore, it is essential to monitor the number of CD34 + cells in the PB every day. Considering the mechanism responsible for the effect of the chemotherapy regimens on bone marrow leading to stem cell mobilization, clear evidence exists only for cyclophosphamide (CY). Many studies have been conducted in humans, primates, and mice that showed release of active proteases in the bone marrow in response to G-CSF and CY[80,100]. The proteases cleave and inactivate many proteins that hold HSCs within the bone marrow stroma. CY increase the release of neutrophil proteases in the BM, with cleavage of VCAM-1 and decreased SDF-1a concentration in the BM. Winkler *et al*[101] demonstrated that CY induced a major reduction in SD-F1a mRNA ex-pression that promoted HSC mobilization without impairment of kit-ligand expression, indicating maintenance of niche functions and rapid recovery afterward. In addition, they observed a reduction in endosteal osteoblasts, bone formation, and F4/80+ osteomacs, while osteoid remained on the endosteum despite the absence of osteoblasts.

One of the often administered regimens is an intermediate dose of CY at 2-4.5 g/m^2, whereas high doses at 7 g/m^2 have been used as well, followed by the administration of G-CSF at a dose of 5-10 μg/kg/d[102]. Others used etoposide in combination with CY and/or cisplatin or added paclitaxel and concluded that the regimens were more effective for stem cell mobilization than CY alone. Moreover, Weaver *et al*[103] in 1998, used taxanes, either paclitaxel or docetaxel, in combination with CY, followed by G-CSF, and observed more efficient mobilization, almost three times more efficient than CY + G-CSF alone in patients with metastatic breast cancer[103].

The most frequently used regimen in patients with GCTs is paclitaxel at 200 mg/m^2 on day 1 plus ifosfamide at 2 g/m^2/d on days 1-3 (TI) supported with G-CSF at 10 μg/kg/d, starting on day 4[104,105]. TI was shown by Rick *et al*[104] more efficient than TI with the addition of cisplatin; *i.e.* the TIP regimen. An interesting mobilization regimen was used in the TAXIF study, wherein the epirubicin was added to paclitaxel. Despite the different chemotherapy mobilization regimens that have been used, the most commonly applied are TI or TIP, as was shown in a retrospective study by Hamid *et al*[106] (see also Table 1 for detailed references to the studies).

REMOBILIZATION STRATEGIES

Dose escalation of cytokines

Higher doses of G-CSF agents have been suggested as a strategy to improve mobilization and peripheral stem cell collection, but the evidence is conflicting. Some studies found no significant difference when a dose of 5 μg/kg/d was administered compared with the most broadly applied doses of 10 μg/kg[107,108]. Similarly, twice daily administrations did not demonstrate improved stem cell yields[109]. However a number of studies conducted in hematologic patients, provided compelling evidence that higher doses improved mobilization.

Structural modifications to improve poor physicochemical properties

Lenograstim: Lenograstim, a glycosylated form of G-CSF, also widely used for HSC transplantation, was hypothesized to induce increased mobilization compared to conventional G-CSF agents. In fact, it was proposed that its unique structure and glycosylation pattern provided protection against elastase-dependent inactivation, and could thereby lead to prolonged activity and increased mobilization[110,111]. Several studies though did not find any differences on HSC mobilization with collection results and patient outcomes comparable to conventional G-CSF-mobilized patients. Therefore, data on its efficacy remains to date both limited and inconclusive[112-114].

Pegfilgrastim: Pegfilgrastim is a pegylated form of G-CSF with long half-life characteristics because of its significantly reduced renal excretion[115]. It promotes stem cell mobilization with a single dose administration, as opposed to the daily injections of the regular short half-life G-CSF[116,117]. The results of recent studies have been controversial, as a number of them supported a significant increase in peripheral stem cells collected, while others found no difference in terms of stem cell mobilization, when a double dose of 12 mg-compared to the 6mg dose after conventional chemotherapy-was administered[118].

Table 1 Clinical studies applying various hematopoietic stem cell mobilization chemotherapy + granulocyte colony-stimulating factor protocols in patients with relapsed/refractory germ cell tumors

Ref.	Number of patients	Successful mobilization	Mobilization regimen
Fruehauf *et al*[149] 1995 (prospective analysis)	15	Median BM 31.49 × 10^6/kg PB 0.46 × 10^6/kg 100%	Cisplatin 100 mg/m^2 etoposide 75 mg/m^2 ifosfamide 2 g/m^2 + G-CSF
Tada *et al*[150] 1999 (retrospective analysis)	6	2.5 × 10^8/kg 100%	Cisplatin 200 mg/m^2 ifosfamide 4 g/m^2 etoposide 100 mg/m^2 d1-d3 + G-CSF
Rodenhuis *et al*[151] 1999 (multicenter prospective phase II)	35	10.3 × 10^6/kg 100%	Cisplatin 200 mg/m^2 ifosfamide 4 g/m^2 etoposide 100 mg/m^2 d1-d3 + G-CSF
Lotz *et al*[152] 2005 TAXIF 2005 (retrospective analysis)	45	9 × 10^6/kg (for 3 HDCT) 100%	Epirubicin 120 mg/m^2 - paclitaxel 200 mg/m^2 + G-CSF
Argawal *et al*[102] 2009 (retrospective analysis)	37	3-6 × 10^6/kg 100%	ifosfamide 2-4.5 g/m^2 + G-CSF
Feldman *et al*[153] 2010 (prospective phase I/II)	107	> 2 × 10^6/kg 100%	TI: paclitaxel 200 mg/m^2 d1 ifosfamide 2 g/m^2 d1-d3 + G-CSF
Haugnes *et al*[154] 2012 (prospective analysis)	882	> 2 × 10^6/kg 100%	BEP-ifosfamide + G-CSF
Mohr *et al*[155] 2012 (retrospective analysis)	44	> 4 × 10^6/kg 100%	PEI (cisplatin, etoposide, ifosfamide) + G-CSF Plerixafor in poor mobilizers
Necchi *et al*[156] 2015 (review)	42	> 2 × 10^6/kg 100%	BEP + G-CSF
Moeung *et al*[157] 2017 (pharmacokinetic phase II study)	89	> 9 × 10^6/kg (for 3 HDCT) (1-2 cycles) 100%	TI: paclitaxel, ifosfamide + G-CSF
Hamid *et al*[106] 2018 (retrospective analysis)	35	10/35 plerixafor + G-CSF 95%	TI: paclitaxel, ifosfamide or TIP
Argawal *et al*[158] 2019 (retrospective analysis)	321	172 allogeneic 95% 149 autologous 73% 77/149 without plerixafor → 64% success 72/149 with plerixafor → 82% success	G-CSF ± Plerixafor
Yildiz *et al*[159] 2020 (retrospective analysis)	50	> 2 × 10^6/kg 100%	TIP + G-CSF
Ussowicz *et al*[160] 2020 (retrospective analysis)	18 (children)	Median: 4.56 × 10^6/kg 100%	Cyclophosphamide 4 g/m^2 + G-CSF
Chevreau *et al*[161] 2020 (multicenter prospective phase II)	89	> 9 × 10^6/kg (for 3 HDCT) 100%	TI: paclitaxel, ifosfamide + G-CSF

G-CSF: Granulocyte colony-stimulating factor; HDCT: High-dose chemotherapy; TIP: Paclitaxel (Taxol)-ifosfamide-cisplatin.

Addition of mobilizing agents affecting a different pathophysiological pathway in order to improve peripheral stem cell collection

Ancestim: Ancestim is a recombinant human SCF that, through its binding to the c-kit receptor on HSCs, modulates their proliferation and adhesion, and has shown promising synergy in HSC mobilization when combined with G-CSF[119,120]. Limited efficacy when administered alone has also been noted[119]. Unfortunately, data available from recent studies did not confirm the efficiency in enhancing chemotherapy or growth factor-induced PBSC mobilization in patients with a prior insufficient PBSC collection, thus, limiting its further application[121].

GM-CSF: GM-CSF and its synergistic effect when combined with chemotherapy are no longer in use because the superiority of G-CSF in terms of mobilization and safety profile has been proved in a number of studies (*e.g.*, faster neutrophil recovery and fewer transfusions required)[122,123]. GM-CSF is sometimes used in combination with G-CSF in patients who failed an initial mobilization attempt, as a second or even as a third agent[124], despite the fact that several studies reported that the association of the two cytokines was not superior to G-CSF alone[125].

Plerixafor (Mozobil): Briefly, plerixafor was first studied as an agent against HIV [126]. During those clinical trials, neutrophilia was observed that sparked numerous studies[127]. In December 2008, plerixafor was approved by the Federal Drug Administration for use with G-CSF for HSC mobilization and collection and subsequent

ASCT in patients with non-Hodgkin lymphoma (NHL) and multiple myeloma (MM), who had failed prior mobilization with G-CSF alone or chemotherapy + G-CSF (plerixafor: AMD3100). The first report of the use of plerixafor in heavily pretreated, refractory and relapsed patients with GCTs was by Kobold *et al*[128]. Plerixafor was given subcutaneously in combination with G-CSF at a dose of 240 μg/kg after at least 4 d of G-CSF, which was given at the standard dose of 10 μg/kg/d. Plerixafor was administered 6 to 11 h before apheresis when a PB CD34+ count higher than 10/μL was achieved. The combination was successful, and allowed collection of sufficient numbers of CD34+ cells in 67% of the patients who failed prior mobilization with chemotherapy and G-CSF[128].

Despite the fact that the efficacy of plerixafor as a stem cell mobilization agent in patients with GCTs undergoing HDCT and ASCT has been reported in a number of small patient series and case studies, its use has not yet been approved, because of the lack of prospective studies. Thus, the indications for the use of plerixafor as a mobilization agent in patients with relapsed/refractory GCTs are not yet clear and rely on the opinions of the authors who published the studies (see Table 2 for details).

Structure and mechanism of action are as follows. Plerixafor (or AMD3100) is a bicyclam derivative that reversibly competes with and inhibits SDF-1a binding to CXCR4. CXCR4 is expressed on many cell types including white blood cells, epithelial, endothelial cells, and HPCs. It plays a critical role in the homing and trafficking of HPCs, as well as their retention and maintenance in the bone marrow niche. CXCR4 is a member of one of the two major families of chemokines. Chemokines are defined by the number and spacing of cysteine residues at the N-terminal end of the protein. CC cytokines have two cysteine residues that are adjacent; in CXC cytokines they separated by one amino-acid residue[129]. CXCR4 ligand, the chemokine SDF-1a (CXCL12), is produced by bone marrow stromal cells including osteoblasts, endothelial cells, and adventitial cells. Plerixafor was shown to directly inhibit SDF-1a ligand binding, SDF-1 mediated G-protein activation, calcium flux, and receptor internalization[130]. In another study, Lee *et al*[131] described the activation of phosphorylation of MAPK-p42/44 in granulocytes and monocytes by plerixafor, which induced the secretion of several proteases from the cells and enhanced the cleavage and activation of C5 in plasma. The C5 cleavage fragments (C5a and desArgC5a) play a critical role, as mentioned earlier, in the egress of HSCs. Granulocytes, stimulated and chemo-attracted by these fragments, enhance secretion of proteolytic enzymes that perturb HSCs retention signals and help HSCs to move through the endothelial barrier[131].

A possible mechanism for plerixafor-stimulated HSCs mobilization was proposed by Dar *et al*[132], in which an increase in CXCL12 circulating in the plasma was observed after the administration of plerixafor. At the same time, CXCL12 levels in BM fluids were decreased. The changes correlated with an increase of circulating progenitor cells in the blood, suggesting that SDF-1 actively regulated the number of circulating progenitor cells. Furthermore, the plasma levels of S1P, a potent chemoattractant for hematopoietic progenitors, was increased following AMD3100 administration[132].

The pharmacokinetics of plerixafor after subcutaneous injection show a peak plasma concentration within 30-60 min. Up to 58% of plerixafor is bound to plasma proteins, and it is eliminated by the urinary route with a half-life of 4 h. Similar increases in HSC levels are observed after multiple daily injections, suggesting no cumulative drug effect after consecutive injections[37,38]. An interesting fact about the timing of plerixafor injection and the mobilization of CD34+ was reported by Lefrere *et al*[38]. They found that in good mobilizers, the PB CD34 + count remained high for at least 12 h after G-CSF plus plerixafor administration[38]. In contrast, in poor mobilizers, precise monitoring of the PB CD34+ cell count was required, because the peak CD34+ cell count occurred 6-9 h after plerixafor injection[38]. It is essential to emphasize the significant decrease in CD34+ count that was observed in the patients 8-12 h after the injection, in order to determine the optimal timing of apheresis[38]. Regarding adverse effects, plerixafor is well tolerated, with rare reports of severe side effects, such as hypotension, dizziness, and thrombocytopenia. The most commonly observed adverse effects are diarrhea, nausea, and skin erythema at the injection site [38].

Future novel approaches: Most novel HSC mobilizing agents are initially tested in MM and NHL patients, and ASCT candidates. Successful application in that setting allows further testing in patients with relapsed/refractory GCTs and other solid tumors where HDCT and autografting are indicated at some point during the disease course. CXCR4 antagonists like plerixafor, emerged as potent agents to rescue "hard-

Table 2 Clinical studies applying plerixafor with granulocyte colony-stimulating factor ± chemotherapy for hematopoietic stem cells mobilization in patients with relapsed/refractory germ cell tumors

Ref.	Number of patients participating	Successful mobilization rates on previously failed chemotherapy + G-SCF driven mobilization (> 2 × 10^6)	Mobilization techniques
Kobold *et al*[128] 2011 (Retrospective analysis)	6	66.67% (4)	Chemo + G-CSF failed
			Plerixafor + G-CSF
Horwitz *et al*[162] 2012 (Retrospective analysis)	21	76% (17)	Chemo + G-CSF failed
			Plerixafor + G-CSF
Worel *et al*[163] 2012 (Retrospective analysis)	11	91% (10)	Plerixafor + G-CSF
Garcia-Escobar *et al*[164] 2014 (Case series)	5	80% (4)	Chemo + G-CSF failed
			Plerixafor + G-CSF
Kosmas *et al*[165] 2014 (Pilot study)	14 (3)	100% (3)	Chemo + G-CSF failed
			Chemo + Plerixafor + G-CSF
O'Hara *et al*[166] 2014 (Retrospective analysis)	9 (3)	100% (3)	Plerixafor + G-CSF

Related case studies: Saure *et al*[167], 2010; Tuffaha and Adel-Rahman[168], 2011; De Blasio *et al*[169], 2013; Miltiadous *et al*[170], 2017. G-CSF: Granulocyte colony-stimulating factor.

to-mobilize" patients with MM, NHL, GCTs, and some rare solid tumors. Research in that area has expanded with the development of novel CXCR4 inhibitors, such as motixafortide (BL-8040) and BKT140 (4F-benzoyl-TN14003), a 14-residue biostable synthetic peptide that binds CXCR4 with much greater affinity than plerixafor (84 nmol/L *vs* 4 nmol/L). An interim analysis of the phase 3 GENESIS trial of motixafortide *vs* placebo, both with G-CSF, for HSC mobilization in MM demonstrated an almost 4.9-fold increased efficacy in obtaining the primary endpoint of a target of 6.0 × 10^6 CD34+ cells/kg with up to two apheresis sessions and that 5.6-fold more patients achieved that target with one apheresis. Moreover, the motixafortide arm allowed 88.3% of patients to proceed to transplant, as opposed to 10.8% in the placebo arm [133]. Another peptide CXCR4 antagonist, a clinical stage compound balixafortide (POL6326) was evaluated in healthy volunteers and proved to be safe, well tolerated, and induced effective mobilization of HSCs at doses ≥ 1500 μg/kg and was predicted to yield an adequate collection of 4 × 10^6 CD34+ cells/kg in a single apheresis[134].

Another area of interest in HSC mobilization is the role of the sphingosine-1-phosphate/S1P receptor 1 (S1P/S1P1) axis, and studies in mice demonstrated an additional PB HSC mobilization benefit of S1P1 agonist (SEW2871) treatment in combination with a CXCR4 antagonist, but not human G-CSF[135]. However, that approach still remains experimental, with no apparent clinical testing so far.

Small molecule inhibitors of VLA-4 such as BIO5192 and monoclonal IgG4 antibodies (*e.g.*, natalizumab) bind to the a4 subunit of the a4β1 (VLA-4) integrin expressed on most leucocytes including CD34+ progenitor cells, inhibit the interaction of VLA4 primarily with VCAM-1 (CD106) on stromal cells, and secondarily with other ligands, including the segment-1 domain of fibronectin[136,137]. The interactions lead to increased HSCs in the blood. Therefore, their application has been proposed in patients with hematologic malignancies who are candidates for ASCT[138,139]. Unfortunately the clinical use of VLA-4 inhibitors is currently limited to multiple sclerosis and other inflammatory diseases.

Bortezomib (Velcade, PS-341) is a proteasome inhibitor that interferes with the activation of nuclear factor-kappa B (NFκB) by preventing proteasomal degradation of IκBa. VCAM-1 expression is upregulated by the VCAM-1 promoter. The latter is activated by binding to NFκB6. As proteasome inhibitors can indirectly inhibit transcription and expression of VCAM-1, and knowing the importance of the VCAM1-VLA4 interaction for HSC homing and mobilization, the application of proteasome

inhibitors as a mobilizer of HSC was proposed[140].

Hypoxia-inducible factor (HIF) prolyl hydroxylase (PHD) inhibitors, such as FG-4497, synergize with G-CSF and plerixafor to enhance mouse HSC mobilization. Deletion of the Hif1a gene weakens the effect[141]. A potential mechanism of FG-4497 proposed in recent studies includes stabilizing HIF-1a protein and increased VEGF-A secretion by BM macrophages[64,65]. FMS-like tyrosine kinase-3 Ligand (FLT3L) binds the FLT3 (CD135) receptor expressed on HSCs and induces proliferation, differentiation, development, and mobilization. Its efficacy has been shown either as a single agent, or in combination with other molecules mentioned above, such as IL-8 or G-CSF [142]. As chemokine-chemokine receptor axes are involved in retention of HSCs in the BM microenvironment, chemokine receptor agonists have been proposed as therapeutic agents to facilitate the mobilization process. The compounds include agonists of the CXCR4 receptor expressed on HSCs (*e.g.*, CTCE-0021 and ATI-2341)[143] or chemokines binding to chemokine receptors expressed on granulocytes and monocytes [*e.g.*, CXCL2, also known as the growth-related oncogene protein-beta (GRO-β) and its specific binding to the CXCR2 receptor; CCL3, also known as macrophage inflammatory protein-1α (MIP-1α); or CXCL8, also known as IL-8, could be used alone or in combination with other mobilizing agents like G-CSF or plerixafor (AMD3100)][144-146].

A novel mobilization strategy was developed and tested in mice through combined targeting of the chemokine receptor CXCR2 on granulocytes and VLA4 in HSCs. Treatment resulted in rapid and synergistic mobilization along with an enhanced recruitment of long-term repopulating of HSCs. That was achieved when a CXCR2 agonist, a truncated form of GRO-β; (tGRO-β) was administered in conjunction with a VLA4 inhibitor, leading to rapid and potent HSC mobilization, which represents an exciting potential strategy that warrants clinical development[147]. A G-CSF-free mobilization regimen using a tGRO-β compound, MGTA-145, which is a CXCR2 agonist, in combination with plerixafor was developed in the context of *in vivo* HSC transduction as a gene therapy approach in a mouse model of β-thalassemia[148]. The MGTA-145+plerixafor combination resulted in robust mobilization of HSCs. Importantly, compared with G-CSF + plerixafor, MGTA-145 + plerixafor led to significantly less leukocytosis and no elevation of serum interleukin-6 levels, and was thus likely to be less toxic[148]. However, the above regimen has not yet been tested for HSCs mobilization in neoplastic diseases. Therefore, evidence is accumulating that CXCR4 receptor agonists could be used with other agents as mobilizing drugs. In particular, they may provide an alternative for patients who are poor mobilizers.

CONCLUSION

Despite the fact that GCTs are currently considered as curable tumors, almost 30% of patients presenting with metastatic disease at diagnosis are likely to experience disease progression at some point. The use of HDCT and ASCT has been established as a salvage therapeutic option, but a number of patients fail to mobilize with conventional strategies. Such poor mobilizers endanger the safety of the procedure. Along with conventional mobilization strategies, such as G-CSF and chemo-mobilization, the use of newer mobilizing agents like plerixafor has emerged with promising results for this group of patients.

Algorithms to improve the efficiency of HSC mobilization, for example "just in time" and preemptive, aim to minimize failures, obtain the desired CD34+ HSCs dose for one or more transplants with the least apheresis sessions, and thus reduce overall healthcare costs, are urgently required. As novel HSC mobilizing agents are initially tested in preclinical experimental models and hematologic malignancies, such as NHL and MM, their application in solid tumors, candidates for ASCT, and in particular GCTs, is lagging behind.

Two axes responsible for HSC retention in the BM stroma that have been explored are the CXCR4-CXCL12 (SDF-1) and the VLA4 (α4/β1)-VCAM1 pathways. Novel inhibitors of those interactions have been evaluated, either alone or in combination with G-CSF, or with GRO-β/CXCR2 axis co-stimulation. Nevertheless, as studies in this area are limited, future investigation should concentrate on finding new agents or establishing proper mobilization algorithms to achieve an adequate CD34+ dose required for a successful ASCT.

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REVIEW

Re-irradiation for high-grade gliomas: Has anything changed?

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Abstract

Optimal management after recurrence or progression of high-grade gliomas is still undefined and remains a challenge for neuro-oncology multidisciplinary teams. Improved radiation therapy techniques, new imaging methods, published experience, and a better radiobiological knowledge of brain tissue have positioned re-irradiation (re-RT) as an option for many of these patients. Decisions must be individualized, taking into account the pattern of relapse, previous treatment, and functional status, as well as the patient's preferences and expected quality of life. Many questions remain unanswered with respect to re-RT: Who is the most appropriate candidate, which dose and fractionation are most effective, how to define the target volume, which imaging technique is best for planning, and what is the optimal timing? This review will focus on describing the most relevant studies that include re-RT as salvage therapy, with the aim of simplifying decision-making and designing the best available therapeutic strategy.

Key Words: Re-irradiation; Recurrent glioma; High-grade gliomas; Glioblastoma; Radiosurgery; Stereotactic radiotherapy

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Core Tip: The optimal management after recurrence or progression of high-grade gliomas is still undefined. Improved radiation therapy techniques, new imaging methods, published experience, as well as better radiobiological knowledge of the brain tissue have positioned re-irradiation as a valid alternative for many of these patients. Many questions remain unanswered. This review will focus on describing the most

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relevant studies that include re-irradiation as salvage treatment, with the aim of simplifying decision-making and designing the best available therapeutic strategy.

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INTRODUCTION

High-grade gliomas (HGG) are the most common primary malignant brain neoplasm in adults[1]. The most frequent type, glioblastoma multiforme (GBM), has an incidence of 3 cases/100000 inhabitants[2]. Its treatment is a macroscopically complete tumor resection, whenever possible, followed by external beam radiotherapy (60 Gy in 2 Gy/fr) with concurrent temozolomide (TMZ) and adjuvant TMZ until the completion of six cycles[3]. Nevertheless, approximately 40% of World Health Organization (WHO) grade III gliomas (anaplastic astrocytoma) and 90% of grade IV gliomas (GBM) progress within 2 years. The main site of relapse is in or near the tumor bed[3-5].

With standard treatment, median overall survival (mOS) for GBM is approximately 14.6 mo, and median progression-free survival (mPFS) is 6.9 mo[6]. This tumor has a poor prognosis and is very aggressive and fast-growing. The high rate of local failure suggests secondary therapeutic options for local salvage should be considered.

The first issue during the diagnostic-therapeutic approach is to confirm that we are dealing with true tumor progression. The phenomena of "pseudoprogression", described in 20%-30% of patients who have received radiochemotherapy and possible radionecrosis (RN), associated or not with tumor, may hinder or delay diagnosis[7]. The Response Assessment in Neuro-Oncology working group criteria[8] for HGG categorization has certain limitations.

Optimal management after recurrence or local progression remains to be defined. It has mostly been established by retrospective studies lacking a quality of life (QoL) evaluation. Established salvage treatment options include a second surgery (re-S), re-irradiation (re-RT), systemic treatment, or some combination thereof[9]. The addition of the "tumor treating field therapy" approach (alternating electrical fields that exert biophysical force on charged and polarizable molecules known as dipoles) has been found to extend survival for patients with newly diagnosed and recurrent GBM (rGBM)[10].

These suboptimal results have motivated multiple lines of research investigating new therapeutic approaches such as the addition of molecular targeted agents, immune checkpoint blockade, vaccines, viral therapy, or other irradiation modalities[11-14].

Current therapeutic approaches, including the radiation therapy techniques and parameters, are very diverse. Thus, a survey of expert radiation oncologists showed high variability, reflecting the scarcity of high-quality prospective data for decision-making[15]. Multiple questions remain unanswered with respect to re-RT: Who is the most appropriate candidate, which dose and fractionation are most effective, how to define the target volume, and which imaging technique is best for planning, as well as the optimal timing? This review will focus on describing the most relevant studies that include re-RT as salvage therapy, with the aim of simplifying de-cision making and designing the best available therapeutic strategy.

RE-RT IN THE THERAPEUTIC STRATEGY

At present, any ablative treatment option offered to a selected patient with local failure is still palliative and has associated side effects that must be considered. The choice is complex, and the criteria are poorly defined. Decisions must be individualized, taking into account the pattern of relapse, previous treatment, and functional status, as well as the patient's preferences and expected QoL.

For patients with low functional status, unable to walk and totally dependent for daily activities, the best supportive care should be considered.

Historically, the fear of exceeding the dose tolerance of healthy brain tissue, and therefore the risk of severe side effects, kept radiation oncologists from considering re-RT with ablative doses. Thus, the most offered treatment has been systemic [chemotherapy/bevacizumab (BEV)], with a mOS of 6-9 mo, without a clear advantage of any drug or therapeutic scheme among those used[16,17]. Clearly this is the best strategy for patients with widespread or multifocal disease. However, in the case of a focal relapse, if the patient has favorable clinical criteria, the current trend is to consider a second local treatment such as re-S, re-RT, or both with or without systemic treatment.

The level of evidence supporting this approach is low, probably because the high failure rates (recurrence or progression) of these second treatments make it difficult to compare the different strategies.

Objective parameters are needed to simplify therapeutic decision-making. Scoccianti *et al*[18], based on a review of the literature, recommend the first algorithm to aid decision-making in daily practice between surgical salvage or re-RT. They consider local treatment for focal relapses in patients with life expectancy > 3 mo. The choice of re-S or re-RT depends on prognostic factors and the expected toxicity of each therapeutic option. The results of combined treatment are encouraging, and the tendency is to recommend it. The therapeutic decision should be interdisciplinary and requires expert neurosurgeons and radiation oncologists. Ultimately, the final decision should be agreed upon with the patient after discussion of the risks and benefits of the available therapeutic options.

RESULTS

Re-resection

A minority of patients (20%-30%) are considered eligible for re-S[19], with a higher morbidity-mortality than before initial resection. After re-S, overall survival from re-RT ranges from 4.9[20] to 13.5 mo[21] and PFS from re-RT from 1.9[22] to 8.3 mo[23]. These results are from retrospective, not comparative series. There is no evidence to suggest that these results are better than can be expected with radiation and/or chemotherapy alone[24,25]. The meta-analysis of Lu *et al*[26] suggests that re-S of rGBM in select patients confers a significant, prognostic OS advantage independent of other prognostic factors, and a cohort from The Director Trial[27] found that surgery at first recurrence of GBM improved outcome if complete resection of contrast-enhancing tumor was achieved. Preoperative and postoperative Karnofsky performance status (KPS), extent of surgery of first re-S, and chemotherapy after first re-S have been identified as the factors that have the greatest impact on survival[25].

Due to the absence of comparative studies, the role of re-S in rGBM is not yet established. The Randomized Controlled Comparative Phase II Trial on Surgery for Glioblastoma Recurrence trial comparing re-S of recurrence plus second-line treatment, *vs* second-line treatment without re-S, will quantify the contribution of re-S for rGBM.

Re-RT

Based mostly on retrospective series, selected patients with small recurrent tumors and a good performance status may benefit from re-RT using modern high-precision techniques[28-31]. Prospective studies are very scarce, therefore the exact contribution of re-RT is uncertain.

The tumoricidal dose to be administered is limited by the possibility of generating severe side effects, given that most patients have already received doses in the maximum tolerance range at their first irradiation. Re-RT at the therapeutic doses used at diagnosis (60 Gy) is not recommended.

Potential benefits of re-RT include palliation by reducing corticosteroid use, improving neurologic symptoms, and, in selected patients, increasing PFS and possibly overall survival.

There are three most commonly used external radiation therapy techniques that, depending on the fractionation applied, the treatment volume, and the technology used we refer to as: Stereotactic radiosurgery (SRS), hypofractionated stereotactic radiotherapy (HFSRT), and conventionally fractionated external radiotherapy (CFRT). We also have results with intraoperative techniques[32]. The promising results of particle irradiation are described in the section on new irradiation strategies. The

choice of technique, in addition to its geographical availability, depends on the size of the recurrence and consequently of the planning target volume (PTV) generated.

Unfortunately, the lack of comparative trials does not allow their results to be compared. However, even in the absence of randomized data, there is a tendency to use hypofractionated or SRS schemes for small volumes, assuming a slightly higher risk of RN.

Kazmi *et al*[33] published the first meta-analysis with the results of re-RT in rGBM. They included 50 studies with a total of 2095 patients. Overall survival from re-RT and PFS from re-RT at 6 mo were 73% and 43%, respectively, and at 12 mo were 36% and 17%. They found better PFS at 6 mo with SRS and with short fractionation schedules (≤ 5 fractions), probably due to the lower tumor volume.

SRS as salvage treatment

Table 1 describes a selection of series published since 2005. They are characterized by: Including mostly GBM, a single dose of 12-18 Gy, a median volume of around 10 mL, and a time from the first radiation treatment of between 8.8 mo and 13.8 mo. The Kong series[34] is the largest and the only prospective series. The mOS for GBM is between 7.5 mo and 13 mo, while the range for mPFS, in those series that report it, is between 3.6 mo and 7 mo. Severe toxicity is not reported, except for RN, which in a couple of series is 24%-31% by radiological imaging. These data suggest that patients with small volumes can be safely treated with SRS.

Fractionated stereotactic radiotherapy as salvage treatment

Hypofractionated schemes have been used mainly in larger recurrent HGG (rHGG). A selection of studies published in 2000 or later, including several prospective series, are presented in Table 2. Some contain anaplastic and low-grade gliomas. The median dose and fractionation used are highly variable, between 25 and 35 Gy (3-7 Gy/fr), with an equivalent dose at 2 Gy (EQD2) range of 37.5-78.7 Gy. The largest series is Fogh *et al*[29] with 147 patients, of which 42 had anaplastic astrocytomas, with an average dose of 35 Gy (3.5 Gy/fr) and a mOS of 11 mo for rGBM. Severe toxicity is also highly variable, with some series reporting none and others as much as 10.5% and a percentage of radiological RN between 6%-11%.

A recent study, in a large and heterogeneous series of 198 patients with rHGG, reports a mOS of 7 mo (6 mo for GBM and 14 mo for grade III gliomas) with good tolerance. The most common fractionation schedules were 41.8 Gy-49.4 Gy/3.8 Gy/fr [35].

The main study with CFRT is by Combs *et al*[36]. They analyzed 59 patients with rGBM treated with 36 Gy/2Gy/fr, achieving an mOS of 8 mo, with only 1.7% of histologically confirmed RN despite a large median tumor volume (49.3 mL). This indicates that it may be an adequate schedule in larger lesions.

Several retrospective papers have compared the different techniques (SRS, HFSRS, CFRT), reporting similar results between them, with mOS of 9.7-11 mo[37,38].

There are very few prospective studies on the efficacy of re-RT *vs* systemic treatment alone. RTOG 0525[39] has reported mOS of 8.2 mo with re-RT, 10.5 mo with chemotherapy, and 11.3 mo with radiochemotherapy. Patients who only received best supportive care had an mOS of 4.8 mo, probably selected for worse overall status. Available data in rGBM generally suggest that re-RT modestly improves PFS compared with systemic treatment alone, but OS is similar[40].

Re-RT of larger volumes

The main hurdle for re-RT of voluminous relapses has been the risk of RN. Most re-RT studies describe a PTV < 40 mL[41,42]. The available evidence for large volume lesions is sparse and few studies include a median PTV greater than 75 mL. Two authors report the largest series to date. The study by Scholtyssek *et al*[43], with a median PTV of 110.4 mL and doses of 36 Gy (30 Gy-40.05 Gy) at 2-5 Gy/fr, did not describe severe toxicity or RN. Chan *et al*[44], with a median PTV of 145.3 mL and dose of 35 Gy/15 fr, in 67 patients, reported 4 cases of radiological RN. The mOS reported in these series were 7.7 and 7.8 mo, in the same range as reported in studies with small treatment volumes. We can conclude that re-RT of large volume disease is feasible, provided that the doses administered are appropriate.

Re-RT with concurrent systemic treatment

Two drugs (TMZ, BEV) are mainly used. Although they have been shown to be safe combinations, their benefit has yet to be demonstrated.

Table 1 Summary of selected publications reporting radiosurgery as salvage treatment in recurrent high-grade gliomas

Ref.	Study type	No. patients	Histology	Re-irradiation		Median interval	Median tumor volume	Median PFS2	Median OS2	Severe toxicity	Radionecrosis
				Total dose, median	Dose/fr, median						
Combs *et al* [28], 2005	R	32	All GBM	15 Gy	63.8 Gy	10 mo	10 mL	7 mo	10 mo	0%	0%
Hsieh *et al* [104], 2005	R	26	All GBM	12 Gy	42 Gy	NR	21.6 mL	NR	10 mo	NR	31.3% by image
Kong *et al* [34], 2008	P	114	65 GBM, 49 G3G	16 Gy	72 Gy	NR	10.6 mL	4.6 mo (GBM), 8.6 mo (G3G)	13 mo (GBM), 26 mo (G3G)	0%	24.4% by image
Patel *et al* [68], 2009	R	26	All GBM	18 Gy	90 Gy	12.5 mo	10.4 mL	NR	8.4 mo	Limited toxicity	NR
Skeie *et al* [30], 2012	R	51	All GBM	12.2 Gy	43.3 Gy	11 mo	12.4 mL	6 mo	12 mo	0%	0%
Martínez-Carrillo *et al*[31], 2014	R	87	46 GBM, 41 G3G	18 Gy	90 Gy	13.8 mo	8.7 mL	NR	7.5 mo (GBM); 17 mo (G3G)	0%	0%
Kim *et al* [105], 2015	R	29	All GBM	15 Gy	63.8 Gy	8.8 mo	11 mL	3.6 mo	9.2 mo	NR	NR

α/β = 2; EQD2: Equivalent dose at 2 Gy fractions; G3G: Grade III glioma; GBM: Glioblastoma; NR: Not reported; OS2: Overall survival from re-irradiation; P: Prospective; PFS2: Progression free survival from re-irradiation; R: Retrospective.

Re-RT with TMZ: Table 3 summarizes the main results. The techniques used have been HFSRS or CFRT. Hematologic ≥ grade 3 toxicity of up to > 40% has been described. RN has been reported, either radiological (7%-8%)[45,46] or histopathological in 4.3%[47]. The mOS for GBM ranges from 9.7-14 mo[45,48] and mPFS between 4-7 mo[46,47], results reported without the combination of TMZ. However, in the Grosu *et al*[49] and Conti *et al*[47] series, patients receiving TMZ had higher mOS.

Overall, concurrent approaches with TMZ do not appear to improve re-RT outcomes and may carry increased risk of toxicity. However, these findings need to be confirmed in prospective series.

Re-RT with BEV: The association of BEV to treatment with first line radiochemotherapy did not demonstrate a benefit in OS in two phase III trials[50,51]. In recurrences, the role of concurrent BEV with re-RT is still not well defined, but several studies have confirmed the safety of this combination with reasonable survival results [52-57]. Table 4 summarizes the main results. The mOS ranges between 9.3-12.2 mo for rGBM, with mPFS between 5.2 and 6.8 mo. This combination has been shown to decrease the risk of RN, especially for re-RT of larger volumes[44,58]. The percentage of symptomatic RN/symptomatic edema, defined as the need for corticosteroids > 6 wk after re-RT, was lower with the BEV combination (21.8% *vs* 37.8%, $P = 0.025$), with these differences increasing at 1 year (23.9% *vs* 54.1%, $P = 0.013$).

The highly anticipated results from the NRG Oncology/RTOG 1205 phase II clinical trial (NCT01730950) are expected in 2023. It randomizes patients with recurrence to BEV alone or BEV with concurrent re-RT (35 Gy in 10 fractions for tumors smaller than 5 cm). Preliminary results of this study have confirmed the safety of the BEV-Re-HFSRT combination and that it provides a benefit in PFS at 6 mo, even without a benefit in mOS, as observed in first line.

Re-RT after progression to BEV

Recently, a new scenario of re-RT has been explored, after progression to BEV. Several groups have published data on this approach, showing an mOS of 5.4 mo[59] and 4.8 mo[39]. The combination of minocycline, BEV, and fractionated re-RT after progression to BEV has been investigated in a phase I trial[60]. PFS3 was 64.6%, and mOS was 6.4 mo. This study adds a prospective trial to the literature showing that re-RT of HGG after BEV failure can be performed with acceptable tolerability. Another recently published phase I trial included 32 patients with rHGG and the combination of

Table 2 Summary of selected publications reporting hypofractionated stereotactic radiosurgery as salvage treatment in recurrent gliomas

Ref.	Study type	No. patients	Histology	Re-irradiation			Median interval	Median tumor volume	Median PFS2/actuarial PFS2	Median OS2	Severe toxicity	Radionecrosis
				Total dose, median	Dose/fr, median	EQD2						
Selch *et al*[106], 2000	R	21	15 GBM, 3 G3G, 2 G2G, 1 no biopsy	25 Gy	5 Gy	43.8 Gy	11 mo	12 mL	5 mo	6.7 mo	0%	0%
Vordermark *et al* [107], 2005	R	19	9 GBM, 10 G2G	30 Gy	5 Gy	52.5 Gy	19 mo	15 mL	4.9 mo, 4.6 mo (GBM)	9.3 mo, 7.9 mo (GBM)	10.5% other than necrosis	0%
Ernst-stecken *et al* [108], 2007	P	15	10 GBM, 3 G3G. 2 G2G	35 Gy	7 Gy	78.7 Gy	10 mo	22.4 mL	15 mo	12 mo	20% need to increase steroids dose without evidence of progressive disease	NR
Fokas *et al*[78], 2009	P	53	All GBM	30 Gy	3 Gy	37.5 Gy	NR	35 mL	22% at 12 mo	9 mo	0%	0%
Fogh *et al*[29], 2010	R	147	105 GBM, 42 G3G	35 Gy	3.5 Gy	48.1 Gy	8 mo	22 mL	NR	11 mo (GBM)	0.7% toxicity (severe headaches)	0%
Mckenzie *et al*[69], 2013	P	33	29 GBM, 4 G3G	30 Gy	5 Gy	52.5 Gy	NR	8.54 mL	62% at 6 mo	8.6 mo	9% toxicity other than necrosis	9% by image
Ogura *et al*[80], 2013	R	30	15 GBM, 9 G3G. 6 G2G	35 Gy	7 Gy	78.7 Gy	NR	9 mL	3 mo	10.2 mo	13.3% need to increase steroids dose without evidence of progressive disease	6.1% by image
Miwa *et al*[109], 2014	P	21	All GBM	30 Gy	5 Gy	52.5 Gy	12 mo	27.4 mL	NR	11 mo	4.8%	9.5%
Dincoglan *et al* [110], 2015	R	28	All GBM	25 Gy	5 Gy	43.8 Gy	11.2 mo	36.5 mL	5.8 mo	10.3 mo	0%	11% G2 by image

α/β = 2; EQD2: Equivalent dose at 2 Gy fractions; G: Grade; G2G: Grade II glioma; G3G: Grade III glioma; GBM: Glioblastoma; NR: Not reported; OS2: Overall survival from re-irradiation; P: Prospective; R: Retrospective; PFS2: Progression free survival from re-irradiation.

pembrolizumab, an anti-programmed cell death protein 1 (PD-1) monoclonal antibody, HFSRT, and BEV, with an mOS and mPFS of 13.4 mo and 7.9 mo, respectively. The authors concluded that this combination is safe and well tolerated, meriting further investigation[61].

Re-resection and re-radiation therapy

Straube *et al*[62] was the first author to suggest that this strategy could be beneficial, after concluding that the pattern of relapse in 26 patients with complete re-S was solely local in 70%. Based on this, and taking into account the maximal safe resection, several groups have demonstrated the value of additional re-RT with different techniques[38,

Table 3 Summary of selected publications reporting re-irradiation plus temozolomide as salvage treatment in recurrent high-grade gliomas

Ref.	Study type	No. patients	Histology	Re-irradiation			Median interval	Median tumor volume	Median PFS2/actuarial PFS2	Median OS2/actuarial OS2	Severe toxicity	Radionecrosis
				Total dose, median	Dose/fr, median	EQD2						
Grosu *et al*[49], 2005	P	44 (TMZ 29)	34 GBM, 2 Gliosarcomas, 8 G3G	30 Gy	5 Gy	52.5 Gy	16 mo	15 mL	NR	8 mo (11 mo RT + TMZ *vs* 6 mo without TMZ)	0%	0%
Combs *et al*[81], 2008	R	25	8 GBM, 10 G3G, 7 G2G	36 Gy	2 Gy	36 Gy	36 mo	50 mL	5 mo; 16% at 12 mo	8 mo; 25% at 12 mo	0%	NR
Minniti *et al*[45], 2011	R	36	All GBM	37.5 Gy	2.5 Gy	42.2 Gy	14 mo	13.1 mL	5 mo; 8% at 12 mo	9.7 mo; 33% at 12 mo	Thrombocytopenia G3: 2.8%	8% by image
Conti *et al*[47], 2012	R	23 (TMZ 12)	All GBM	20 Gy	10 Gy	60 Gy	7 mo	< 30 mL	7 mo (TMZ) *vs* 4 mo (no TMZ)	12 mo (TMZ) *vs* 7 mo (without TMZ)	≥ G3 hematological toxicity > 40%	4.3%
Minniti *et al*[46], 2013	R	54	38 GBM, 16 G3G	30 Gy	6 Gy	60 Gy	15.5 mo	9.8 mL	6 mo (4 mo GBM)	12.4 mo (11.4 mo GBM)	Thrombocytopenia G3: 3.7%, leukopenia G3: 3.7%	7% by image
Greenspoon *et al*[111], 2014	P	31	All GBM	30 Gy	5 Gy	52.5 Gy	At least 6 mo	12 mL	7 mo	9 mo	NR	G3: 9.6%, G4: 3.2%
Aktan *et al*[48], 2015	R	21 (17 TMZ)	18 GBM, 3 G3G	54 Gy	2 Gy	54 Gy	39.4 mo	Recurrent tumor size was median 5.5 cm	NR	18 mo (G3G) and 14.1 mo (GBM)	0%	0%

α/β = 2; EQD2: Equivalent dose at 2 Gy fractions; G2G: Grade II glioma; G3G: Grade III glioma; G: Grade; GBM: Glioblastoma; NR: Not reported; OS2: Overall survival from re-irradiation; P: Prospective; R: Retrospective; PFS2: Progression free survival from re-irradiation; TMZ: Temozolomide.

63-65].

Combs *et al*[63] published the first study after rHGG re-S followed by early re-RT. It included 108 patients, most of whom received 36 Gy at 2-3 Gy *per* fraction. The mOS was 12 mo, with no serious toxicity. In multivariate analysis, the extent of surgery, methylguanine-DNA methyltransferase (MGMT) methylation, interval time between first and second irradiation, and KPS were independent prognostic factors for OS. A subsequent study[64], with 25 interventional rGBM cases treated with HFSRS and simultaneous integrated boost (37.5 Gy and 45 Gy in 15 fractions), reported an mPFS of 13 mo and mOS of 16 mo, with better outcomes in smaller recurrences, without eloquent area involvement and in patients with a good general condition.

On multivariate analysis, the macroscopic tumor volume (GTV) ≥ 100 mL *vs* < 100 mL was confirmed as an independent prognostic factor affecting OS. Radiologically suspected RN was observed in 16 patients (64%) at a median of 9 mo after re-RT, and 8 patients developed grade 3 RN requiring hospitalization.

Table 4 Summary of selected publications reporting re-irradiation plus bevacizumab as salvage treatment in recurrent high-grade gliomas

Ref.	Study type	No. patients	Histology	Re-irradiation			Median interval	Median tumor volume	Median PFS2	Median OS2	Severe toxicity	Radionecrosis
				Total dose, median	Dose/fr, median	EQD2						
Gutin *et al* [56], 2009	P	25	20 GBM, 5 G3G	30 Gy	6 Gy	60 Gy	14.5 mo	34 mL	7.3 mo	12.5 mo	G3: 1 hemorrhage; G4: 3 (1 bowel perforation, 1 wound dehiscence and 1 GI bleed)	0%
Cuneo *et al* [54], 2012	R	63 (41 BEV)	49 GBM, 8 G3G, 6 prior G2G	15 Gy	15 Gy	63.8 Gy	21 mo	4.8 mL	GBM: 5.2 mo (BEV) *vs* 2.1 mo (without BEV). 6 mo whole series	GBM: 11.2 mo (BEV) *vs* 3.9 mo (without BEV). 10 mo whole series	11%	10%
Niyazi *et al* [52], 2012	R	30 (20 BEV)	22 GBM, 8 G3G	36 Gy	2 Gy	36 Gy	NR	NR	8 mo	Mean 12 mo	G3:1; G4: 1 wound dehiscence	0%
Shapiro *et al* [112], 2013	R	24	20 GBM, 1 G3G, 3 G2G	30 Gy	6 Gy	60 Gy	12.6 mo	35.3 mL	7.5 mo (6.8 mo GBM)	12.2 mo (whole series and GBM)	Toxicity BEV: G4: 12.5%	0%
Cabrera *et al* [113], 2013	P	15	8 GBM, 7 G3G	18 Gy. 25 Gy	18 Gy. 5 Gy	90 Gy. 43.8 Gy	20 mo	NR (< 5 cm)	3.9 mo	14.4 mo	G3:1	0%
Flieger *et al* [57], 2014	P	71 (57 BEV)	52 GBM, 19 G3G and G2G	36 Gy	2 Gy	36 Gy	NR	NR	5.6 mo (BEV) *vs* 2.5 mo (without BEV)	GBM: 9.3 mo (BEV) *vs* 6.1 mo (without BEV)	Toxicity BEV: G4: 5.3%	4.2% (BEV) by image or histologically

α/β = 2; BEV: Bevacizumab; EQD2: Equivalent dose at 2 Gy fractions; G: Grade; GBM: Glioblastoma; G2G: Grade II glioma; G3G: Grade III glioma; NR: Not reported; OS2: Overall survival from re-irradiation; P: Prospective; PFS2: Progression free survival from re-irradiation; R: Retrospective.

In the series by Chun *et al*[65] with 84 patients, the addition of radiation therapy (median dose of 45 Gy at 1.8 Gy/fr) to re-S was associated with a significant benefit in PFS, with mPFS for re-S being 3.5 mo and 9 mo for re-S plus re-RT. The benefit in OS was marginal, with an mOS of 12.7 mo with re-S *vs* 28.1 mo with re-S plus re-RT (P = 0.066). Three risk factors (age ≥ 50, WHO grade IV, and unmethylated promoter of MGMT) were significantly associated with poor OS in multivariate analysis. The authors established three categories of risk groups based on these factors. The benefit of re-RT in both OS and PFS was established in patients with two or more risk factors (intermediate and high risk groups). There was no radiological or pathological evidence of RN during or after re-RT.

Results of the GlioCAVE/NOA 17 trial (NCT02715297) should better determine the contribution of early adjuvant radiotherapy after re-S in rGBM. It is a prospective phase II study with a schedule of 46 Gy at 2 Gy/fr or 36 Gy at 3 Gy/fr, with PFS as the primary endpoint.

Prognostic scales for re-RT

The first scale to predict OS after re-RT published by Combs[66] derived from 233 patients with recurrent low- and HGG and included: WHO grade, age at the time of re-RT, and the time interval to re-RT. The same group published a modified version, the New Combs Score, which added other factors such as KPS, tumor volume, and re-S prior to re-RT[67]. This new revalidated scale[38,63] with a simple approach, is practical, useful, and widely used for decision making (Table 5).

Other reported prognostic features include the re-RT dose[31,57], use of salvage chemotherapy[43,57], extent of resection[28,43], MGMT promoter methylation status [46], and radiographic response[68-70]. However, how they should be quantified remains to be described.

Interestingly, Chapman *et al*[38], without finding an association of irradiation technique (SRS *vs* non SRS) or fractionation with survival, identified a threshold dose as a function of PTV size that should not be exceeded to minimize toxicity: 40 Gy Biological Equivalent Dose 10 for SRS (16 Gy in 1 fraction) and 45 Gy Biological Equivalent Dose 10 for non-SRS treatments (approximately 30 Gy in 5 fractions, 35 Gy in 10 fractions or 40 Gy in 20 fractions), from the same range as those identified in other series[29,57,71]. And, globally, it identifies a group of patients who can achieve an advantage in OS and PFS with re-RT, in particular young patients with good KPS, longer time interval from initial radiation to first progression, small recurrence volume, and an adequate re-RT dose.

RADIOTHERAPY SPECIFICATIONS

Treatment volumes

Definition of target volumes: The definition of re-RT target volumes should be conservative, minimizing the irradiation of healthy tissues to avoid severe toxicities (RN). It requires not only extreme precision and conformality during treatment but also precise images that identify the exact location of tumor tissues. Inaccuracies in tumor delineation may diminish any gain in local control achieved by dose escalation. One aspect to consider would be whether the relapse is located in the area of previous maximum dose or is marginal or remote from the first irradiation. In this case, and depending on the volume of the relapse, the dose prescription can be less conservative.

Several studies have shown that standard anatomic imaging modalities [computed tomography, magnetic resonance imaging (MRI)], while very accurate in visualizing normal anatomic structures, are limited in defining the exact extent of the tumor. Classically, volume delineation for irradiation is based on T1-weighted MRI with gadolinium. Contrast uptake is a consequence of blood-brain barrier disruption and does not necessarily reflect the actual tumor extent in gliomas. Macroscopic tumor masses far from the margins of contrast enhancement have been detected in surrounding edema and even in adjacent normal-appearing brain tissue[72-74]. Anti-angiogenic drugs may also condition contrast uptake, as they may initially have a stabilizing effect on the blood-brain barrier[75].

Multiple studies correlating imaging findings with histopathologic evaluation in surgically treated patients with HGG have indicated that molecular imaging with amino acid positron emission tomography (PET) is more specific and equally sensitive for tumor detection than MRI (T1 with gadolinium). Grosu *et al*[49] have postulated that target volumes for re-RT should be based on amino acid PET imaging in addition to MRI, to include the actual tumor dimension. Other imaging modalities have been used to delineate GTV, including spectroscopy MRI, perfusion-weighted imaging and diffusion-weighted imaging[76], 11C-methionine PET[49], and 18 F-dihydroxyphenylalanine PET[46]. However, there are no randomized trials that have evaluated the impact of molecular or functional imaging-based radiotherapy on the outcomes achieved.

The ongoing phase II GLIAA (NOA 10/ARO 2013-1) trial[77] is the first randomized study evaluating the impact of differences in planning volumes designed with molecular *vs* MRI imaging on PFS after re-RT in patients with rGBM. The limited availability of molecular and/or functional imaging equipment together with the lack of evidence of its superiority in the design of planning volumes conditions the continued use of MRI images for re-RT volume definition.

Volumes-exclusive radiotherapy: The definition of the target volume generally includes the GTV, defined as any contrast-enhancing lesion on T1-weighted MRI. In most studies, the clinical target volume (CTV) equals GTV[28,29,78]. Some papers add

Table 5 Scoring scheme and new prognostic groups of the "New Combs Score"

Prognostic factors	Prognostic value
Primary histology	
Glioblastoma, WHO IV	2
Anaplastic glioma, WHO III	1
Low-grade glioma, WHO I/II	0
Age	
≥ 50 yr	1
< 50 yr	0
Time between primary RT and re-RT	
≤ 12 meses	1
> 12 meses	0
Re-resection performed	
No	1
Yes	0
KPS	
< 80%	1
≥ 80%	0
Tumor volume (PTV)	
> 47 mL	1
≤ 47 mL	0
Scoring group	Scoring value/mOS
a	0–1/19.5 mo
b	2–3/11.3 mo
c	4–5/8.1 mo
d	6–7/5.5 mo

KPS: Karnofsky performance status; mOS: Median overall survival; PTV: Planning target volume; RT: Irradiation; WHO: World Health Organization.

a CTV to include the peritumoral edema visualized in the fluid-attenuated inversion recovery sequence of the MRI, since it is known that tumor cells can be found in this location[79,80]. Subsequently, a margin usually ≤ 5 mm is added for PTV expansion [45,49,78], although some authors include up to 1 cm[57,81].

Volumes-adjuvant radiotherapy: For re-S patients, Straube *et al*[62] proposed a GTV including the resection cavity and contrast enhancement areas, with a margin of 5-10 mm to generate the CTV and 1-3 mm to create the PTV. The GLIOCAVE-NOA 17 study[82] meets these criteria. The CTV encompasses the margins of the resection cavity, including all areas of contrast enhancement plus 5 mm.

DOSAGE AND FRACTIONATION

The optimal dose and fractionation schedule in these patients is unknown. Re-RT is a well-known factor contributing to the risk of RN, which is directly associated with dose and irradiated volume.

Sminia and Mayer[83] examined > 25 glioma re-RT studies to assess tolerance dose and treatment volume of normal brain tissue. RN occurred with a cumulative EQD2 dose ($\alpha/\beta = 3$) > 100 Gy for CFRT, > 105 Gy for fractionated stereotactic radiotherapy (FSRT), and 135 Gy for SRS.

Given that these patients have already received 60 Gy after initial diagnosis, there is a margin of at least 40 Gy for re-RT. Hence, the prescribed doses for re-RT in most published studies ranged from 30-45 Gy, thus maintaining a cumulative EQD2 of approximately 100 Gy[64]. However, given that brain tissue recovers over time, it seems safe to administer higher doses to smaller volumes, using FSRT or SRS, without increasing the likelihood of RN[83].

Scoccianti *et al*[42], after an extensive review of published series and always proposing schemes with reported severe toxicity ≤ 3.5%, described a treatment strategy depending on the volume to be irradiated. Thus, for small volumes (≤ 12.5 mL) SRS schemes are safe (*e.g.*, 12-15 Gy) provided that the EQD2 value does not exceed 65 Gy; HFSRT (*e.g.*, 5 × 5 Gy) for medium-sized lesions (> 12.5-35 mL), provided that the EQD2 value does not exceed 50 Gy and CFRT (*e.g.*, 36 Gy in 20 fr) for larger lesions (> 35-50 mL). These authors pointed out that this recommended strategy should be confirmed in prospective studies.

Whenever possible, hypofractionated schemes are preferred, avoiding unnecessary transfers in these patients with limited life expectancy.

Organ-at-risk tolerance dose

In primary treatment, the maximum doses to the brainstem, chiasm, and optic nerves to avoid the risk of myelopathy are well defined[84,85]. In the context of re-RT in HGG, current evidence is limited[15,44]. Preclinical data suggest a 61% recovery in the spinal cord after 1 year since the first irradiation, and it is believed that this is likely to be applicable to other central nervous system tissues[86]. These models indicate that, in the context of re-RT, maximum summed doses of up to 86 Gy could be tolerated for the optic chiasm and brainstem.

Two series with low recorded toxicity analyzed cumulative dose in organ-at-risk with different doses and fractionations. Shen *et al*[71] reported a median maximum dose in the brainstem of 76.9 Gy and 56 Gy in the optic pathway, with a CFRT schedule and a mean dose of 41.4 Gy. In the series of Chan *et al*[44], with a dose mostly of 35-40 Gy/15 fr, the median maximum dose was 64 Gy for the brainstem and 54.9 Gy for the optic chiasm, although it is noted that concomitant BEV was administered, which may reduce the risk of RN.

It is essential to record and communicate doses to organ-at-risk before re-RTs in order to be able to design a toxicity risk model.

TOXICITY AND QOL

Toxicity

Data on re-RT toxicity are scarce in the literature (Tables 1-4), and its analysis and quantification are difficult. Late toxicity assessment is limited by poor prognosis, difficult differentiation between tumor recurrence, and RN, which is associated with the variety of techniques and fractionations used.

The only existing meta-analysis[33] reported a grade ≥ 3 toxicity rate of 7%, and the morbidity and mortality rate for re-RT ranged from 0%-31% and 0%-1%, respectively.

QoL

Disease progression is associated with deterioration of neurocognitive function. The evidence supporting treatment in this population is evolving, but little is known about its impact on QoL. The survival benefit is desirable but must be carefully weighed against expected morbidities.

Analysis of pooled data from over 300 GBM patients from 13 published articles showed that overall, re-RT resulted in clinical improvement in 24%-45% of patients and a reduction in corticosteroid dependence in 20%-60% of patients. However, the subgroup with KPS < 70 appeared to have a higher risk of early progression and apparently had less benefit from re-RT[87].

Very few studies prospectively evaluate the impact on QoL and activities of daily living in the setting of salvage re-RT. Wick *et al*[88] analyzed QoL in 84 patients with rGBM from a phase II trial with Asunercept/APG 101 and re-RT *vs* re-RT alone, with a dose of 36 Gy at 2 Gy/fr. The EORTC QLQ-C15-PAL, EORTC QLQ-BN20, and Medical Research Council scale questionnaires were used, concluding that Asunercept plus re-RT significantly prolonged time to deterioration of QoL *vs* re-RT alone. More recently, Maitre *et al*[89] reported prospective data on QoL and activities of daily living in patients with recurrent/progressive glioma treated with re-RT (median dose EQD2 51.4 Gy). They used the QLQ-C30 and QLQ-BN20 questionnaires and the modified

Barthel index. They performed 225 evaluations in 60 patients, concluding that high-dose re-RT in selected patients is associated with stabilization of QoL and greater functional independence.

NEW STRATEGIES FOR RE-RT

New re-RT strategies for the treatment of HGG recurrences include particle radiotherapy, as well as intraoperative radiation therapy (IORT) and brachytherapy. Although they are not novel techniques, they are re-emerging in recent years with technological advances.

Particle irradiation

Proton therapy is emerging for the treatment of these patients. Due to its physical and radiobiological properties, this radiation modality offers dosimetric advantages over photons, achieving a better dose distribution and decreasing the irradiation of healthy tissue. The Proton Collaborative Group has published the largest series to date[90]. They analyzed 45 patients with a median of 20.2 mo between initial diagnosis and recurrence. The median dose was 46.2 Gy (range, 25-60 Gy), with a mean of 2.2 Gy/fr, achieving mPFS and mOS of 13.9 and 14.2 mo, respectively. The treatment was well tolerated, and the appreciated toxicity was related to a dose higher than 41 Gy (EQD2). Only prior surgery was positively associated with PFS and OS.

The first study of re-RT with carbon ion beams in rHGG analyzed 30 patients with a median interval between initial radiotherapy and re-RT of 10 mo[91]. The dose administered was 45 Gy in 15 fractions, with a mOS of 13 mo. Eight patients had grade 3 toxicity. Only initial histology with a Ki67 < 20% was a prognostic factor. Resection or chemotherapy did not significantly improve OS. A phase I/II trial to compare re-RT of recurrent gliomas with carbon ions *vs* re-RT with photons is ongoing (NCT 01166308).

IORT

IORT data come from older series, mainly from HGG at diagnosis, and only a few papers included rGBM[32]. The results were promising, but the complexity of the procedure led to abandoning its use. The development of portable systems capable of being moved to the operating room has sparked interest in this technique. This approach is conceptually attractive because it allows the delivery of a large dose of radiation to the tumor bed and tumor debris close to the surgical cavity immediately after resection, while respecting the surrounding brain tissue, decreasing the likelihood of RN. In addition, local and systemic immune responses may be promoted, which could benefit oncological outcomes[92,93].

Recently, although in newly diagnosed GBM, Giordano *et al*[94] reported the results of a phase I/II dose-escalation trial, evaluating the safety and efficacy of the Zeiss INTRABEAM system, a miniaturized 50 keV LINAC with spherical applicators. Fifteen patients, mainly with subtotal resection, were included, receiving a dose of 30 and 40 Gy, with no evidence of limiting toxicity, achieving a PFS of 17.7 mo.

Brachytherapy

Like IORT, it has the advantage of allowing immediate irradiation of the surgical cavity[95], without having to wait the usual 4 wk until the surgical wound is completely healed to start external radiotherapy. This delay is not desirable in HGGs, where in as little as 3 wk there is already a high rate of tumor repopulation. The most commonly used technique is permanent seed implantation. Initially the isotope used was I-125, but high complication rates were reported[96]. Suture-stranded Cs-131 seeds, with a shorter half-life, are now the most commonly used isotope. A study combining re-S with insertion of suture-stranded Cs-131 seeds and BEV (before or after the procedure) has recently been published[95]. Twenty patients were analyzed, with a dose of 80 Gy administered at 0.5 cm from the surface of the resection cavity. Seven patients had been previously salvaged with external radiotherapy. Local control was 85% and mOS was 9 mo. There were two wound infections and three seizures, with no case of RN.

These radiation techniques are safe and effective, but further prospective and comparative research is needed to draw solid conclusions.

SPECIAL PATIENT GROUPS

Elderly patients

As in younger patients, radiotherapy is the cornerstone of first-line treatment of older patients with GBM. However, they receive poor care after recurrence[97]. The evidence for re-RT in older patients is very scarce, as the median age in published papers is around 53 years[33]. However, the aging population is growing and treatment decisions in patients with rGBM and good general condition are increasing. To our knowledge, only one study on re-RT in older patients has been published. Straube *et al* [98] reported the results of 25 patients with a median age of 69.6 years (range 65-79) who received re-RT, most after reintervention. The mOS was 6.9 mo and mPFS at 4.3 mo, with no case of severe toxicity attributable to re-RT. This survival is within the range of series reported in younger patients[28]. Therefore, although prospective trials are needed, these results suggest that second-line salvage therapy should not be dismissed on the basis of age alone.

Pediatric patients

As with adults, children with rHGG have limited treatment options. Re-RT has an emerging role as a palliative treatment for children with recurrent brainstem glioma (diffuse intrinsic pontine glioma or DIPG)[99-101], being associated with symptomatic improvement and longer survival compared to non-re-irradiated patients[99]. Indeed, re-RT in DIPGs is the subject of several ongoing or completed prospective studies (NCT01777633 and NCT03126266). Given that the irradiation dose tolerance of the supratentorial brain is higher than that of the brainstem, it stands to reason that re-RT in supratentorial rHGG should be equally safe and effective[102]. However, the role of re-RT has been little studied in non-pontine gliomas.

Recently, Tsang *et al*[103] have published the results of the largest known cohort of children with recurrent supratentorial HGG treated with re-RT compared to a group of non-re-irradiated children. They retrospectively analyzed 40 patients ≤ 18 years. Fourteen patients, with an interval of at least 6 mo after the first radiotherapy, were re-irradiated. Doses administered ranged from 30-54 Gy at 1.8 Gy/fr. Median survival was 9.4 mo for re-RT patients compared to 3.8 mo for the 26 who did not receive re-RT. The time elapsed between the first and second irradiation determined significant differences, being higher in children with an interval ≥ 12 mo. One patient presented grade 3 RN 4 mo after re-RT. There were no significant differences between patients with initial *vs* distant field re-RT, between those who received concurrent chemotherapy *vs* exclusive re-RT, or between those who were previously operated *vs* those who received radiotherapy alone. Thus, offering re-RT to these patients is associated with reasonable short-term control and survival without significant toxicity.

CONCLUSION

The rHGG scenario remains devastating. Nevertheless, the available evidence, albeit low level, suggests that re-RT, at recommended doses and in selected patients, is safe and provides encouraging local control and survival rates.

The combination of re-S with early re-RT appears to be the most promising option.

Randomized clinical trials are needed to establish the optimal treatment strategy for these patients.

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MINIREVIEWS

Real-world evidence on first- and second-line palliative chemotherapy in advanced pancreatic cancer

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Abstract

In spite of recent diagnostic and therapeutic advances, the prognosis of pancreatic ductal adenocarcinoma (PDAC) remains very poor. As most patients are not amenable to curative intent treatments, optimized palliative management is highly needed. One key question is to what extent promising results produced by randomized controlled trials (RCTs) correspond to clinically meaningful outcomes in patients treated outside the strict frames of a clinical trial. To answer such questions, real-world evidence is necessary. The present paper reviews and discusses the current literature on first- and second-line palliative chemotherapy in PDAC. Notably, a growing number of studies report that the outcomes of the two predominant first-line multidrug regimens, *i.e.* gemcitabine plus nab-paclitaxel (GnP) and folfirinox (FFX), is similar in RCTs and real-life populations. Outcomes of second-line therapy following failure of first-line regimens are still dismal, and considerable uncertainty of the optimal management remains. Additional RCTs and real-world evidence studies focusing on the optimal treatment sequence, such as FFX followed by GnP or vice versa, are urgently needed. Finally, the review highlights the need for prognostic and predictive biomarkers to inform clinical decision making and enable personalized management in advanced PDAC.

Key Words: Pancreatic cancer; Palliative therapy; Cancer chemotherapy; Gemcitabine; Paclitaxel, nano albumin-bound; Folfirinox

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Core Tip: This review summarizes and interprets published real-world evidence of the effectiveness and safety of treatment strategies in advanced pancreatic cancer. The real-world outcomes of first-line chemotherapy regimens such as folfirinox and gemcitabine/ nab-paclitaxel are thoroughly reviewed. The results of randomized controlled trials (RCTs) exploring the regimens seem to be largely generalizable in a real-world context. On second-line options, *i.e.* salvage chemotherapy following failure of first-line therapy, significant uncertainties remain. Additional RCTs and real-world evidence studies addressing current and novel regimens, and the optimal sequence of these, are needed.

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INTRODUCTION

Over the past decades, mortality has decreased for many types of cancer. One exception is pancreatic cancer (pancreatic ductal adenocarcinoma, PDAC), which is soon expected to overtake breast cancer as the second most common cause of cancer-related death[1]. The majority of PDAC patients still present with either locally advanced or metastasized disease, and hence are considered beyond curative potential. For those individuals, as well as for those resected patients who suffer from relapses, palliative systemic therapy and/or radiotherapy are the only treatment options available.

Historically, palliative treatment of PDAC was limited mostly to regimens based on 5-fluorouracil (5-FU), usually with modest results at best. In that setting, 5-FU treatment was more or less experimental, but evidence from a randomized trial in 1996 showed that palliative chemotherapy in PDAC improved median overall survival (mOS) as well as quality of life compared with the best supportive care only[2]. The year after, gemcitabine replaced 5-FU as the gold standard in this clinical scenario based on the results of another randomized trial with prolonged mOS in favor of gemcitabine[3].

For a period of almost 15 years thereafter, many attempts to further improve the treatment in the setting of palliative PDAC were made by adding various cytotoxic drugs and monoclonal antibodies to gemcitabine, often resulting in increased toxicity without any significant survival benefit for patients[4-10]. A first breakthrough came in 2011, when a randomized controlled trial (RCT) showed a significant and clinically meaningful survival benefit over gemcitabine with the triplet combination chemotherapy known as Folfirinox (FFX, 11.1 mo *vs* 6.8 mo for gemcitabine monotherapy)[11]. The survival advantage occurred at the expense of considerably increased hematological and non-hematological toxicity in the intervention group. Another transformative RCT introduced the combination of gemcitabine and nano-albumin-bound paclitaxel (GnP). The regimen produced a smaller effect on overall survival (8.5 mo *vs* 6.8 mo for gemcitabine monotherapy). Nonetheless, it also resulted in increased toxicity, especially myelosuppression and chemotherapy-induced peripheral neuropathy[12]. The finding of more than tripled objective response ratio for the intervention group compared with gemcitabine (roughly 30% *vs* 10%) in both trials indicated a substantial antitumoral effect with the use of those combination regimens. Conversely, the treatment response duration of first-line therapy was usually short, and the RCT-population typically included highly selected patients with lower comorbidity and frailty compared with real-life patients. Whether survival and toxicity data from trials are generalizable to patients treated in routine clinical practice is unclear.

Regarding second-line treatment in PDAC, evidence is scarce. Empirical chemotherapy has been used in highly selected patients, and is usually reserved for very fit or young patients who responded to first-line treatment. Most often, gemcitabine and 5-FU have been used either as monotherapy or in combination with either oxaliplatin or irinotecan. In one of the few RCTs conducted, Oettle *et al*[13]

compared the combination of folinic acid and fluorouracil (FF) in a 42-day cycle with FF in combination with oxaliplatin (OFF). The latter regimen gave significantly longer median progression-free survival (mPFS) and mOS, even though the absolute increase in months was rather small (mPFS 2.0 mo *vs* 2.9 mo, and mOS 3.3 mo *vs* 5.9 mo for FF and OFF, respectively). The occurrence of low-grade neuropathy was more than five times higher (38% of patients) in the OFF group. In contrast, the PANCREOX RCT, which compared the commonly used regimens of 5-FU/leucovorin infusion (5-FU/LV) and modified FOLFOX6 (mFOLFOX6) did not show any advantage with the addition of oxaliplatin[14]. There was no significant difference in the primary mPFS endpoint (3.1 mo *vs* 2.9 mo for mFOLFOX6 and FU/LV). The mOS favored 5-FU/LV (9.9 mo *vs* 6.1 mo for mFOLFOX6). Furthermore, substantial toxicity was observed in the mFOLFOX6-arm, with grade 3-4 adverse events affecting a majority (63%) of participants[14]. A more recent RCT[15] explored the role of 5-FU/LV and liposomal irinotecan in the second-line setting. The combination showed a small survival benefit over 5-FU/LV alone (6.1 *vs* 4.2 mo). However, the 5-FU/LV and liposomal irinotecan combination has not gained widespread traction in countries such as Canada and Sweden because regulatory authorities and health technology assessment bodies have considered the treatment to be not economically justifiable[16-18].

As the results of RCTs may be difficult to interpret and properly implement as standard healthcare, it is essential to complement the basis of knowledge with real-world evidence. The aim of this review was to summarize and assess available studies reporting real-world evidence in support of first- and second-line palliative chemotherapy in advanced PDAC. In first-line therapy, the focus was restricted to the two most established multidrug regimens, *i.e.* FFX and GnP. For second-line therapy, where the evidence on the optimal regimen is weak, no restriction in terms of regimen was applied.

LITERATURE SEARCH

PubMed was searched on December 19, 2020 for studies with titles containing the phrases "pancreatic cancer" and "real world". All results were assessed for potential relevance. Only studies of human pancreatic cancer in the palliative setting and written in English were selected for possible inclusion in this review. Additional requirements for inclusion were information related to chemotherapy (FFX and/or GnP in the first-line setting, or any regimen in the second-line setting); survival [(overall survival (OS) data were required, progression-free survival (PFS) data were optional, and surrogate markers for OS were not accepted); real-world study population, and study type (retrospective or prospective cohort trials). RCTs, published study protocols, case studies, and meeting abstracts were not included. Studies reporting data on several treatment regimens were included as long as either FFX or GnP was among them, and specific survival data and treatment intention for the regimens were clearly distinguishable and compatible with the criteria mentioned above. Included studies are presented in a structured way with key data in tables sorted by topic and year of publication.

RESULTS

The PubMed query on first-line therapy returned 87 publications. Following careful review with regard to the inclusion criteria and scope of this review, 14 articles were selected, four with data on GnP (Table 1), one reporting FFX data (Table 2), eight that compared FFX and GnP (Table 3), and one covering several first-line treatments. The PubMed search of second-line setting returned 17 articles of which 15 were potentially relevant. The articles were subclassified according to which first-line treatment (FFX or GnP) had been administered (Tables 4 and 5). In addition to the above mentioned articles, several papers that did not focus on a specific first- or second-line regimen, and/or described the treatment pattern in general terms, were identified and will be discussed in the relevant section of this review.

First-line GnP combination chemotherapy

Studies evaluating the effect of GnP in the real-world setting are listed in chronological order in Table 1. One study prospectively evaluated the efficacy of the regimen in younger (< 70 years) *vs* older (> 70 years) patients and found no significant between-

Table 1 Real-world studies of gemcitabine/nab-paclitaxel in the first-line setting[19-23]

Ref.	Location	Study design	Stage M1	*n*	Regimen	mOS in mo	Subgroup analysis	mPFS in mo	Remarks
Prager *et al*[19], 2021	Austria	Prospective cohort	100%	317	GnP	10.6/10.2	Age < 70/> 70	5.6/5.5	No difference in frequent toxicities
Blomstrand *et al*[20, 21], 2019/2020	Sweden	Retrospective cohort	71%	75	GnP	10.9	Alb <3 7, age < 65 with shorter survival	5.2	Less hematotoxicity than MPACT
Ostwal *et al*[22], 2018	India	Retrospective cohort	83%	78	GnP	11.6		5.6	Grade III-IV toxicity 35%
Quinton *et al*[23], 2018	United Kingdom	Retrospective cohort	100%	74		8.4		-	Hematotoxicity similar to MPACT

GnP: Gemcitabine/Nab-paclitaxel; M1: Metastatic disease; mOS: Median overall survival; mPFS: Median progression-free survival.

Table 2 Real-world studies of Folfirinox in the first-line setting[24]

Ref.	Location	Study design	*n*	M1	Regimen	mOS in mo	mPFS in mo	Remarks
Cavanna *et al*[24], 2019	Italy	Retrospective cohort	50	74%	FFX/mFFX	10.1	5.6	mFFX sign less toxicity

FFX: Folfirinox; M1: metastatic disease; mFFX: Modified Folfirinox; mOS: Median overall survival; mPFS: Median progression-free survival.

group differences of either mOS (10.6 mo and 10.2 mo) or adverse events[19]. Real-world survival outcomes were superior to those observed in the phase III MPACT trial [12]. The authors suggested that the difference could be explained by a larger fraction of patients proceeding to second-line treatment, 47.4%-56.2% in the real-world studies compared with 38% in the MPACT study[12]. It is also noteworthy that the proportion of patients with performance status (PS) 0 or 1 or corresponding Karnofsky score was somewhat higher than in the MPACT trial. Another study retrospectively evaluated the benefit of GnP in advanced PDAC and found an mOS of 10.9 mo in the entire cohort, and an mOS of 17.1 mo in the locally advanced group[20]. Hematological toxicity was less frequent than in the MPACT study. In the same cohort, multivariate analysis found that low albumin (< 36 g/L) and age (< 65) were significant predictors of worse survival[21].

An additional study found comparable survival outcomes with the use of non-cremophore-based paclitaxel and gemcitabine, with an mOS of 11.6 mo and an mPFS of 5.6 mo[22]. In this retrospective cohort, the majority of patients had metastatic disease (83%) and PS 1 (80%). Grade III-IV toxicity was reported in 36% of patients, with hematological toxicity as the most frequent type of adverse event. In another retrospective cohort analysis where all patients had metastatic disease, mOS was 8.4 mo[23]. Most patients were in PS 1 (66%) at the time of treatment initiation. Similar frequencies of hematological toxicity were seen, with grade III-IV neutropenia being the most frequently reported adverse event (35% of patients).

First-line FFX combination chemotherapy

Studies evaluating the effectiveness of FFX in the real-world setting are listed in chronological order in Table 2. One study evaluated FFX treatment in a retrospective cohort and reported an mPFS of 5.6 mo and an mOS of 10.1 mo[24]. The first 18 consecutive patients received full-dose FFX and the following 32 cases received dose-reduced modified FFX (mFFX), resulting in significantly lower toxicity with fewer hematological and non-hematological side-effects.

First-line FFX vs GnP

Studies comparing the real-world effectiveness of FFX and GnP are listed in chronological order in Table 3. One retrospective cohort study of first-line treatment of patients with metastatic PDAC reported an mOS of 12.7 mo with FFX and 10.2 mo with GnP[25]. Tumor marker serum CA-19-9 and neutrophil-lymphocyte-ratio (NLR) were associated with survival. Authors intended to analyze patients aged above 70 years separately but this group was too small. Hematological toxicity was evenly distributed between the two treatments. Of interest, neuropathy was only reported in

Table 3 Real-world studies comparing Folfirinox and Gemcitabine/Nab-paclitaxel in the first-line setting[25-32]

Ref.	Location	Study design	*n*	M1	Regimen	mOS in mo, *P* value	Prognostic factors	mPFS in mo, *P* value	Remarks
Franco *et al* [25], 2020	Spain	Retrospective cohort	119	50%	FFX 59; GnP 60	FFX 12.7; GnP 10.2; *P* = 0.912	Ca19-9, NLR	-	Toxicity data not reported
Wang *et al* [26], 2019	Canada	Retrospective cohort	225	58%	FFX 92; GnP 87; Gem 46	FFX 14.1; GnP 10.5; Gem 4.2	-	FFX 8.4; GnP 8.5; Gem 3.7	Sign more hematotoxicity in FFX
Pusceddu *et al*[27], 2019	-	Review	3813	NA	FFX 1690; GnP 2123	1.15 longer for FFX. *P* = 0.03	-	-	GnP more neurotoxicity and anemia. FFX more neutropenia
Chiorean *et al*[28], 2019	-	Review	> 6915	NA	FFX > 3556; GnP > 3359	FFX 15.9; GnP 14.4	-	FFX 11.7; GnP 8.5	FFX more neutropenia, GnP more neuropathy
Papneja *et al* [29], 2019	Canada	Retrospective cohort	119	77%	FFX 86; GnP 33	FFX 9.0; GnP 9.0	S-Alb, male sex, 2nd line therapy	FFX 6.0; GnP 4.0	Grade 1-2 thromboembolism, mucositis and neuropathy sign more in FFX. Among grade 3-4 toxicity only fatigue sign more in GnP group
Kordes *et al* [32], 2019	Sweden	Retrospective cohort	595	-	FFX 31; GnP 66; Gem 185	FFX 9.9; GnP 9.8; Gem 6.6	-	-	No sign differences in toxicity comparing FFX *vs* GnP
Cartwright *et al*[30], 2018	United States	Retrospective cohort	486	100%	FFX 159; GnP 255; Gem 72	FFX 11.4; GnP 9.8; Gem 4.4	-	-	No sign differences in toxicity comparing FFX *vs* GnP
Kim *et al* [31], 2018	United States	Retrospective cohort	654	100%	FFX 317; GnP 337	FFX 13.8; GnP 12.1; *P* = 0.96	Age	-	Less toxicity in GnP group

FFX: Folfirinox; Gem: Gemcitabine; GnP: Gemcitabine/Nab-paclitaxel; M1: Metastatic disease; mOS: Median overall survival; mPFS: Median progression-free survival; NA: Not applicable; NLR: Neutrophil-leucocyte ratio; s-Alb: Serum albumin.

two patients receiving FFX. A study that compared the real-world effectiveness of FFX, GnP and gemcitabine reported OS durations of 14.1, 10.5 and 4.2 mo for the three treatments, respectively[26]. FFX treated patients were significantly younger and had better PS, and OS was significantly longer in both FFX- and GnP-treated patients compared with gemcitabine. The majority of patients had metastatic disease (68%). For the subgroups with localized disease, median OS had not been reached at the time of publication. The occurrence of neutropenia, febrile neutropenia, and neuropathy was significantly more frequent in FFX treated patients. In a review article, slightly longer survival (an additional 1.2 mo) was noted in favor of FFX over GnP. Despite the numerical difference, the overall adjusted risk of death was similar regardless of the regimen administered[27]. Neurotoxicity and anemia were seen more frequently in GnP-treated patients; neutropenia was more often associated with FFX treatment. In another review, a similar, non-significant, survival benefit was seen for FFX, with a reported OS of 15.9 mo *vs* 14.4 mo for GnP[28]. PFS was 11.7 mo with FFX and 8.5 mo for GnP. Toxicity data were not consistently reported in the studies, but neutropenia was more often associated with FFX than with GnP. The opposite was observed for neuropathy. In a retrospective study that largely focused on metastatic PDAC patients (77%), equivalent survival for FFX and GnP was reported (OS 9.0 mo for both regimens, P = 0.88). However, PFS was slightly longer with FFX, although the difference was not statistically significant (6.0 mo for FFX *vs* 4.0 mo for GnP, P = 0.38) [29]. There were no significant differences in the frequencies of severe toxicity between the two regimens. Another retrospective study reported OS of 11.4, 9.8 and 4.4 mo for FFX, GnP and gemcitabine monotherapy, respectively. Again, the differences were not significant[30]. Patients receiving GnP were significantly older and had worse PS. Toxicities were evenly distributed between the treatment groups. No significant prognostic factors were found in multivariate analysis, except for PS 2+, which was associated with worse survival. In another Celgene-funded real-world retrospective cohort study, there was a slight, non-significant, trend that favored FFX over GnP, with an OS of 13.8 mo compared with 12.1 mo[31]. All patients had metastatic disease. Common side-effects such as nausea, vomiting, diarrhea and mucositis were less

Table 4 Real-world studies of second-line therapy following failure of Folfirinox[36-44]

Ref.	*n*	M1	2L regimen	mPFS in mo	mOS in mo	Remarks	AE
Portal *et al*[36], 2015	57	100%	GnP	5.1	8.8	Prospective cohort	38% grade 3-4 toxicity
Mita *et al*[37], 2019	30	80%	GnP	3.8	7.6	Phase II	70% grade 3-4 toxicity
Tsang *et al*[38], 2019	159	67%	GnP 78; Gem 81	-	5.8; 4.6	Population-based, three Canadian provinces	-
Zhang *et al*[39], 2018	60	73%; 75%; 73%	GnP 30; Gem 8; BSC 22	3.6; 2.5	5.7; 3.8	Single center	More grade 3-4 fatigue in Gem
Nguyen *et al* [40], 2017	30	77%	GnP	3.7	12.4	Single center	Grade 3-4 thrombocytopenia (33%), anemia (23%), nausea (17%)
Bertocchi *et al* [41], 2015	23	100%	GnP	3.0	5.0	Single center	-
Zhang *et al*[42], 2015	28	82%	GnP	3.0	5.7	Single center	Grade 3-4, anemia (25%), thrombocytopenia (25%), neutropenia (18%)
Caparello *et al* [44], 2016	71	-	GnP	2.5	6.2	Single center	-
Rissy *et al*[43], 2017	12	100%	GnP	4.9	-	Single-center	No grade 3-4 toxicity reported

BSC: Best supportive care; Gem: Gemcitabine; GnP: Gemcitabine/Nab-paclitaxel; M1: metastatic disease; mOS: Median overall survival; mPFS: Median progression-free survival.

Table 5 Real-world studies of second-line treatment with Folfirinox following failure of gemcitabine/nab-paclitaxel or single-agent gemcitabine[46-51]

Ref.	*n*	M1	1L regimen	2L regimen	mPFS in mo	mOS in mo	Remarks
Sawada *et al*[46], 2020	104	100%	GnP	Modified; FFX	3.9	7.0	Bolus 5-FU omitted. 55% grade 3-4 toxicity
Matsumoto *et al* [47], 2020	23	83%	GnP	FFX 12; mFFX 11	5.3; 4.3	6.9; 12.8	No sign difference in toxicity between FFX/mFFX
Assaf *et al*[50], 2011	27	100%	Gem	FFX	3.0	8.5	56% grade 3-4 neutropenia
Kobayashi *et al* [48], 2017	18	100%	Gem	FFX	2.8	9.8	Phase I/II. 83% grade 3-4 toxicity
Kim *et al*[51], 2018	39	82%	Gem	Attenuated; FFX	3.8	8.5	Oxaliplatin: 65 mg/m^2. 41% grade 3-4 neutropenia
Chung *et al*[49], 2018	48	79%	Gem	Reduced irinotecan and oxaliplatin; FFX	5.8	9.0	Phase IIIrinotecan: 120 mg/m^2; Oxaliplatin: 60 mg/m^2; 65% grade 3-4 neutropenia

5-FU: Fluorouracil infusion; FFX: Folfirinox; Gem: Gemcitabine; GnP: Gemcitabine/Nab-paclitaxel; M1: Metastatic disease; mFFX: Modified Folfirinox; mOS: Median overall survival; mPFS: Median progression-free survival.

frequent in the GnP group. A Swedish retrospective study comparing palliative first-line treatment in a PDAC patient cohort that included 31 FFX, 66 GnP, and 185 gemcitabine patients reported OS of 9.9, 9.8 and 6.6 mo, respectively[32]. Patient characteristics, including age and PS, varied substantially among the three groups. No significant differences in grade 3 or higher toxicities were reported between FFX and GnP.

Second-line real-world studies

Second-line treatment in PDAC: Despite advancements in the first-line treatment of advanced PDAC, most patients progress and succumb to the disease. To date, three phase III randomized clinical trials have been reported in the second-line treatment space[13-15] and are thoroughly described above under the background heading.

These three trials compared 5-FU alone *vs* 5-FU/oxaliplatin doublets[13,14] or 5-FU *vs* nal-irinothecan *vs* 5-FU/nal-irinothecan doublet[15], and were all conducted after the patients progressed on gemcitabine-based chemotherapy as first-line treatment for advanced PDAC. However, the contemporary first-line standard treatment includes FFX or GnP combinations for patients with good PS[11,12]. There are no randomized clinical trial data for second-line treatment specifically after failure on FFX and GnP. Second-line treatment of advanced pancreatic cancer is largely driven by the chemotherapy regimen administered in the first-line setting. In a large real-world study that examined the outcome of 167 patients with advanced PDAC using several treatment regimens, the mOS from start of second-line therapy (OS2) was 5.2 mo, and plasma albumin, serum CA-19-9, and performance status were identified as key prognostic factors[33].

Second-line treatment after first-line FFX: In the real world, such patients are usually treated with GnP combination or gemcitabine monotherapy. The initial supportive evidence for use of GnP after first-line use of FFX in advanced pancreatic cancer was published in the form of case reports[34,35]. Subsequently, a prospective multicenter cohort study of 57 patients treated with GnP after FFX failure reported an mPFS of 5.1 mo and an OS2 of 8.8 mo[36]. It is noteworthy that just over half of the patients who received FFX for advanced pancreatic cancer in the frontline setting were eligible to receive salvage therapy with GnP in this cohort study. The objective response rate was 17.5%, while the disease control rate was 58.0%. From the start of first-line chemotherapy, the median OS was 18.0 mo. Grade 3-4 toxicities were observed in 40.0% of patients, of which neutropenia and neuropathy were the two most common. Recently, a phase II study of 30 patients reported in this setting described an mPFS of 3.8 mo and an OS2 of 7.6 mo[37]. The corresponding figures from the start of first-line chemotherapy were 9.3 and 14.2 mo, respectively. The overall response rate was 13.3% and the disease control rate was 46.7%. Grade 3-4 toxicities were reported in 70.0% patients, the most common being neutropenia and neuropathy. Furthermore, several real-world studies have been reported to support the use of GnP as second-line treatment. A large population-based Canadian study compared the real-world data of 368 patients with advanced PDAC treated with first-line FFX across two provinces with differential access to second-line treatment[38]. Of these, 159 patients (43.2%) received second-line treatment that was equally allocated as GnP (49.1%) and single-agent gemcitabine (50.9%). In a secondary analysis, the mOS counted from the initiation of second-line chemotherapy (OS2) was slightly longer for GnP compared with (5.8 mo *vs* 4.6 mo, $P = 0.01$).

Another Canadian study included 60 patients with advanced PDAC who received FFX as the first-line treatment[39]. Of these, 30 patients (50.0%) were treated with GnP, 8 (13.3%) with gemcitabine alone, and 22 patients (37.7%) received optimal supportive care. The mPFS (3.6 mo *vs* 2.5 mo, $P = 0.03$), and OS2 (5.7 mo *vs* 3.8 mo, $P = 0.03$) were longer in patients who received GnP compared with gemcitabine (Table 4). Other real-world studies have reported similar PFS and OS2 with the use of GnP after failure of FFX[40-44]. Furthermore, a recently published systematic review that included 16 studies reported a higher overall response rate (14.4% *vs* 8.4%, $P = 0.038$), disease control rate (53.5% *vs* 30.2%, $P < 0.001$), PFS (3.6 mo *vs* 2.5 mo, $P = 0.030$), and OS2 (5.7 mo *vs* 3.8 mo, $P = 0.030$) with GnP than with gemcitabine monotherapy[45]. Similar grade 3/4 event rates were reported in the prespecified analysis (22.9% *vs* 34.6%, $P = 0.415$). Overall, GnP appears to be a reasonable second-line treatment after FFX and patients considered unfit for GnP may benefit from gemcitabine monotherapy, while those with a poor performance status should be offered the best supportive care.

Second-line treatment after first-line GnP: In the absence of a head-to-head comparison of FFX and GnP in advanced PDAC, a substantial proportion of patients are treated with GnP in the first-line setting. Several chemotherapy regimens using a combination of fluoropyrimidines with irinotecan and/or oxaliplatin have been used in the real-world as salvage, second-line therapy of such patients. It is intuitive to consider FFX in this setting. A recent retrospective analysis of 104 patients treated with modified FFX (*i.e.* intravenous oxaliplatin 85 mg/m^2, intravenous irinotecan 150 mg/m^2, and continuous infusion of 5-fluorouracil 2400 mg/m^2 for 46 h without bolus infusion) in that setting reported an objective response rate of 10.6% and a disease control rate of 56.7%[46]. The median PFS and OS2 were 3.9 mo and 7.0 mo, respectively. Grade 3-4 adverse events were reported in 54.8% patients and included hematological toxicities and peripheral sensory neuropathy. A smaller study of 23 patients who received standard FFX ($n = 12$) and modified FFX ($n = 11$) reported a median PFS of 5.3 mo and an OS of 6.9 mo in patients who received standard dosages.

The corresponding numbers for those receiving modified FFX were 4.3 and 12.8 mo, respectively[47]. The observed differences in survival between the FFX and mFFX groups were not statistically significant.

Other real-world studies have reported the effectiveness of either the standard or modified FFX regimen after failure of single-agent gemcitabine as first-line therapy[48-51]. The studies, which adopted several modifications of the original FFX regimen, reported a PFS of 2.8-5.8 mo and OS2 of 8.5-9.8 mo (Table 2). Overall, limited data from real-world studies supports the use of modified FFX after failure of GnP. However, it is an intensive chemotherapy regimen and a high rate of grade 3-4 adverse events has been reported in above-mentioned studies, primarily hematological events and peripheral neuropathy. Patient selection remains paramount for electing to use such a regimen.

A real-world study of 52 patients with gemcitabine-refractory advanced PDAC reported that nano-liposomal irinotecan with FF was associated with a median PFS of 3.8 mo and OS2 of 6.8 mo[52]. The figures closely mirror the outcome reported from the phase III NAPOLI-1 study[15]. Capecitabine combined with oxaliplatin has also been used in this setting,, and several studies have reported a PFS of around 3 mo and OS2 of approximately 6 mo[53-55]. The median PFS and OS with single-agent capecitabine in 41 patients who failed first-line therapy were reported to be 1.5 mo and 4.3 mo, respectively[56].

Therefore, in patients considered unfit for FFX as second-line treatment, a doublet chemotherapy with fluoropyrimidine and oxaliplatin or nano-liposomal irinotecan is reasonable, while monotherapy with capecitabine may be considered for those with borderline performance status. There are no clinical trials that have compared the efficacy of oxaliplatin with irinotecan in this setting. However, a meta-analysis reported that the combination of a fluoropyrimidine plus irinotecan significantly improved both PFS and OS2, while the oxaliplatin combination modestly improved PFS but not OS2[57]. The modest benefit with these regimens should be balanced with the associated adverse events, and best supportive care should be considered a viable option for patients with poor general condition.

Targeted therapy and immunotherapy

As survival is still short, even when the most effective modern combinations of cytotoxic drugs are administered to patients with good performance status, it is tempting to look for alternatives such as targeted therapies or immune checkpoint inhibitors for the treatment of advanced PDAC. While the major breakthrough is yet to come, some recent findings may have the potential to become game-changing treatments of at least some types of PDAC in the future.

Approximately one in every five patients with advanced PDAC harbors a germline or somatic mutation in the DNA damage repair pathway[58]. There are limited data to suggest that Poly (ADP-ribose) polymerase (PARP) inhibitors may be effective in such patients. For example, a retrospective analysis of patients with previously treated PDAC (median prior therapies = 2) harboring a mutation in the DNA damage repair pathway reported an objective response rate of 23%, PFS of 7.6 mo and OS of 16.5 mo with olaparib[59]. Another report of 30 patients with BRCA1/2 mutations and no available standard treatment options reported disease control rate of 31% and an objective response rate of 4% with olaparib[60]. The role of immunotherapy in advanced PDAC is still evolving. However, a low prevalence (< 2%) of deficient mismatch repair suggests a limited role of immune check point inhibitors in this setting, at least with the currently available drugs[61,62].

CONCLUSION

Pancreatic cancer not amenable to surgical resection remains one of the most difficult challenges for medical oncologists around the globe. Despite improved diagnostic imaging tools, most cases are detected at a stage where cure or long-term survival are not achievable. Nevertheless, there is reason for cautious optimism. Large RCTs over the last decade have introduced first-line FFX and GnP regimens as the current standard of care, which has significantly changed the treatment landscape. Although extrapolation of the outcomes observed in highly selected RCT populations should be done with great care, combined evidence from real-world studies across different countries and health care systems indicates that the regimens are effective and reasonably safe in the real-world setting. In several of the real-world evidence public-

ations, FFX was associated with a slightly better median OS than GnP, but selection bias was probable. Thus, it is possible that the differences observed might be the result of less fit patients being prescribed GnP rather than FFX. A sufficiently large head-to-head RCT comparing first-line FFX and GnP would potentially resolve these issues, but such a study is unlikely to occur.

In terms of second-line therapies, there are still considerable gaps in our knowledge. The few available RCTs provide only limited guidance, and it is difficult to translate their results into real-life practice. Notably, none of the published RCTs addresses whether the sequence of FFX followed by GnP or GnP followed by FFX is the most feasible or beneficial approach. Still, those sequences are often advocated by expert guidelines, and several real-world experience studies support that strategy. The extrapolation of RCTs into the real world is, at least in theory, even more complex in the second-line setting because patients at that point in their disease trajectory are likely to be frailer than patients eligible for first-line therapy.

The accumulating real-world evidence presented in this review points to some key conclusions. Several multidrug regimens show promising potency and acceptable toxicity in the first-line scenario, and to a somewhat lesser extent, the second-line setting. Outcomes reported in RCTs seem to be relatively consistent when the respective regimens are administered in real-life patients. Larger and/or pooled real-world studies are needed to further explore prognostic and predictive parameters such as serum albumin, serum CA-19-9, NLR and other novel biomarkers. Regarding second-line chemotherapy, the RCTs and real-world studies published to date are not fully aligned, and the key question regarding the optimal sequence of regimens remains uncertain. While most patients in this situation have very short expected survival, the identification of reliable clinical and biochemical biomarkers could be very helpful to inform treatment decision making.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Long-term results of the treatment of Hodgkin's lymphoma in a resource-constrained setting: Real-world data from a single center

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Abstract

BACKGROUND

The outcomes of Hodgkin´s lymphoma (HL) in Mexico have not been widely reported. Simplified and affordable treatments have been adopted in middle-income countries.

AIM

The aim was to evaluate long-used therapies for HL in Mexico in a long-term basis.

METHODS

In a 34-year time period, 88 patients with HL were treated at a single institution in Mexico. Patients were treated with adriamycin bleomycin vinblastine and dacarbazine (ABVD) or mechlorethamine, vincristine, procarbazine, and prednisone (MOPP). Relapsed or refractory patients were given ifosfamide, carboplatin, and etoposide (ICE) followed by autologous or allogeneic stem cell transplants.

RESULTS

Informed consent statement: Informed consent was waived by the Institutional Review Board.

Conflict-of-interest statement: The authors declare that they have no conflicting interests.

Data sharing statement: Data are available upon request.

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Thirty-seven women and 51 men were included; the median age was 29 years. Patients were followed for a mean of 128 mo. The 310-mo overall survival (OS) was 83% for patients treated with MOPP and 88% for those treated with ABVD. The OS of patients who received autologous stem cell transplantation was 76% (330 mo) *vs* 93% (402 mo) in those who did not.

CONCLUSION
HL may be less aggressive in Mexican population than in Caucasians. Combined chemotherapy renders acceptable results, regardless of clinical stage.

Key Words: Hodgkin; Lymphoma; Treatment; ABVD chemotherapy

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Core Tip: In a retrospective, observational long-term study, our group found that the treatment of Hodgkin lymphoma in a resource-constrained background may still rely on the use of the traditional adriamycin bleomycin vinblastine and dacarbazine treatment regimen in order to achieve acceptable outcomes. The observations were consistent across different stages of disease and may serve to propose new studies focusing on the comparison of newly approved therapies in contexts where there are some healthcare limitations.

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INTRODUCTION

Hodgkin´s lymphoma (HL) is the model of curative care, with radiation therapy, combination chemotherapy, staging approaches, peripheral blood-stem cell transplantation, and immunotherapy[1]. Following the initial demonstration that radiotherapy could eradicate limited-stage disease, multiagent chemotherapy regimens proved to be curative in a large proportion of patients with advanced disease [1]. In the 40 years since De Vita and colleagues developed the mechlorethamine, vincristine, procarbazine, prednisone (MOPP) chemotherapy regimen, much has been learned about risk stratification to minimize treatment-related toxicity[2]. Doxorubicin (*i.e.* adriamycin), bleomycin, vinblastine, and dacarbazine (ABVD), the most commonly used regimen for both early and advanced stage HL, was developed in the mid-70s[2] and continues to be a standard of care in HL[3]. In recent years, there have been advances, with the introduction of novel therapies and changes in the management algorithms[1]. However, the performance of newer therapies remains unclear in real-world conditions, especially for overall survival (OS) and quality of life of persons with malignant diseases[4]. We analyze here the results of the treatment of a group of 88 patients with HD over a 34-year period at a single institution, treated with combined chemotherapy in a resource-constrained setting of a single institution.

MATERIALS AND METHODS

Patients

All consecutive patients seeking medical care for HL at our institution after 1986 and followed for at least 3 mo were entered into the study. A diagnosis of HL was based on the histological study of a pathology specimen, mainly a lymph node; the same pathologist analyzed all the specimens and defined the histological subtype[5]. The

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clinical stage was defined according to the Ann Arbor classification[5]. Bone marrow biopsies were done only in patients with clinical stages III or IV[6]. Computed tomography (CT) scans were done in all cases prior to starting treatment. Fluorodeoxyglucose positron emission tomography (FDG-PET) scans have been performed since 2002. The study was approved by the institutional review board, and all participants signed an informed consent.

Treatment

Between 1986 and 1997, patients were treated with MOPP, *i.e.* nitrogen mustard (6 mg/m^2 on days 1 and 8 of the cycle), vincristine (1.4 mg/m^2 on days 1 and 8), procarbazine (100 mg/m^2 on days 1 through 14) and prednisone (40 mg/m^2 during cycles 1 and 4 for 14 d)[7]. After 1997, patients were treated with ABVD, *i.e.* doxorubicin (25 mg/m^2), vinblastine (6 mg/m^2), dacarbazine (375 mg/m^2) and bleomycin (10000 units/m^2) on days 1 and 15 of every 4 wk[8] as frontline therapy. Per local protocol, stages I and II, were treated with four cycles of chemotherapy and the response was assessed by a CT scan. If disease activity persisted at that time, four additional cycles were given, whereas two additional cycles were given if the CT scan was negative. For stages III and IV, the CT scans were performed after six cycles, and two or four more cycles were delivered depending on the results, as described above. Bleomycin was administered only in the first three courses and the doxorubicin dose was adjusted to avoid delivering more than 450 mg/m^2. FDG-PET scans have been performed at the end of treatment since 2002. Scans were not performed between cycles. Only patients with disease activity at the end of treatment received radiotherapy. Patients showing activity after treatment were considered as refractory and treated with four courses of ifosfamide, carboplatin and etoposide (ICE)[9]. Autologous or allogeneic peripheral blood hematopoietic stem cell transplant (HSCT) were given to refractory patients after achieving complete remission. High-dose melphalan (200 mg/m^2) was used in autologous transplants; cyclophosphamide, fludarabine, and busulfan were used in allogeneic transplants, which were all from HLA-identical siblings[10]. After the completion of treatment, patients were follow-up every 2 mo for 1 year and every 4 mo from then on. No FDG-PET scans were done during follow-up, unless clinically indicated.

Statistical analysis

The primary outcome measure was OS, defined as the time elapsed between the diagnosis of HL and death from any cause, with censoring of patients who were alive on the last follow-up date. Differences were assessed with Fisher's exact test. OS was estimated by the Kaplan-Meier method, and differences between groups were compared with the log-rank test[11]. Two-sided P values < 0.05 were considered statistically significant. The statistical analysis was carried preformed with Prism 8 (GraphPad Inc. San Diego, CA, United States).

RESULTS

Patient characteristics

Of the 91 patients with HL identified between 1986 and 2020, 88 were followed for 3 mo or more and were included in the analysis. There were 37 women and 51 men. The median age was 29 years (range: 5-73 years). There were 62 patients with nodular sclerosing HL (70%), 19 with mixed cellularity HL, two with lymphocyte depleted HL, and one with lymphocyte predominant HL. In four cases, the histologic variant could not be defined. According to the Ann Arbor classification[5], five patients were stage I, 48 were stage II, 19 were stage III, and 16 were stage IV. Ten patients presented with a mediastinal mass larger than 10 cm in the chest X-ray film. Three cases presented with relapsed disease (Table 1).

Treatment patterns

As frontline therapy, all patients were offered chemotherapy (ChT). Twelve received MOPP and 70 received ABVD; three were treated with initial radiotherapy (RT): two refused ChT, and one was referred after receiving RT. Relapsed or refractory patients were treated with ICE and a subsequent autologous or allogeneic HSCT.

Responses

Patients were followed for a median of 114 mo (range: 4-402). Forty-four are alive, ten

Table 1 Salient features of 88 patients with Hodgkin´s lymphoma

Sex	Women	37 (42)
	Men	51 (57.9)
Age, yr	Median 29 (range: 5-73)	
Type	Nodular sclerosing	62 (70)
	Mixed cellularity	19 (21.5)
	Lymphocyte depleted	2 (2.2)
	Lymphocyte predominant	1 (1.1)
Stage	Stage I	5 (5.6)
	Stage II	48 (54.5)
	Stage III	19 (1.5)
	Stage IV	16 (18.1)

Data are *n* (%)

have died, and 34 were lost to follow-up. Median OS for all patients has not been reached, and is more than 402 mo. OS was 88% 310 mo and 77% 402 mo (Figure 1). Median OS has not been reached and is 94 mo for stage I, 109 mo for stage II, 90 mo for stage III, and 98 for stage IV (P = 0.2). The 310-mo OS was 83% for patients treated with MOPP and 88% for those treated with ABVD [hazard ratio (HR): 0.76, 95% confidence interval (CI): 0.2-2.8, P = 0.6; Figure 2]. Sixteen patients (18%) were refractory to treatment and nine (10%) relapsed. They were treated with ICE followed by HSCT, autologous in 15 patients and allogeneic in ten patients. Patients who underwent autologous HSCT had a median survival of 329 mo and an OS of 92%. Those given allogeneic HSCT had a median survival of 59 mo and an OS of 46% (HR: 0.2, 95%CI: 0.04–1.3, P = 0.057). The OS of patients given and HSCT was 73% at 266 mo and was 93% at 404 mo in those not given HSCT (HR: 4.09, 95%CI: 1.0–16.6, P = 0.01) (Figure 2B). The OS was similar (Figure 2). The causes of death were breast carcinoma in two cases, liver carcinoma in one, and uncontrolled lymphoma activity in the remaining patients.

Long-term toxicity

Twelve patients developed peripheral neuropathy. There were no reported cases of pulmonary, fertility, or cardiovascular toxicity. Five patients developed a secondary neoplasia 18-150 mo after completing treatment; four had received chemotherapy (three ABVD and one MOPP); one had received radiotherapy alone. The salient features of the patients are shown in Table 2.

DISCUSSION

The outcomes of HL in Mexico have not been fully analyzed or reported. We have previously shown that some malignancies in Mexico have different behaviors in the population of Mexican mestizos compared with other populations. For example, multiple myeloma in Mexico is less frequent[12,13] and less aggressive than in Caucasians or African-Americans[12], and chronic lymphocytic leukemia is also less frequent and less aggressive in Mexican mestizos than in other populations[14-16], but promyelocytic leukemia is substantially more frequent in Mexico than in Caucasian populations[14,17]. In the case of HL in Mexico, there is not enough information about its prevalence and clinical behavior. Preliminary data indicate that the prevalence of HL in Mexico is similar to that reported in other populations[17,18]. The data presented here suggest that the clinical picture of the disease may be less aggressive in Mexico, as without employing novel and sophisticated drugs, the OS of this group of patients was 88% at 310 mo, including all stages of the disease, relapsed, and refractory patients (Figure 1). There were no significant differences between patients treated with MOPP or ABVD. The OS of relapsed or refractory patients who were given an HSCT was significantly lower than those who did not require it (Figure 3).

Table 2 Salient features of patients who developed a secondary malignancy after the treatment of lymphoma

	Neoplasm	Age	Sex	Type	Stage	Treatment	HSCT	Time[1], mo
1	Tongue epidermoid carcinoma	32	M	Nodular sclerosing	I	Radiotherapy	No	58
2	Liver adenocarcinoma	22	F	Nodularsclerosing	III	MOPP	No	97
3	Breast cancer	38	F	Mixed cellularity	III	ABVD	No	150
4	NK/T-cell lymphoma	15	M	Nodular sclerosing	IV	ABVD	Autologous	18
5	Breast cancer	23	F	Nodular sclerosing	III	AVBD	Autologous	59

[1]Time in months elapsed between the diagnosis of Hodgkin's lymphoma and the diagnosis of the secondary neoplasia.
HSCT: Hematopoietic stem cell transplant; M: Male; F: Female.

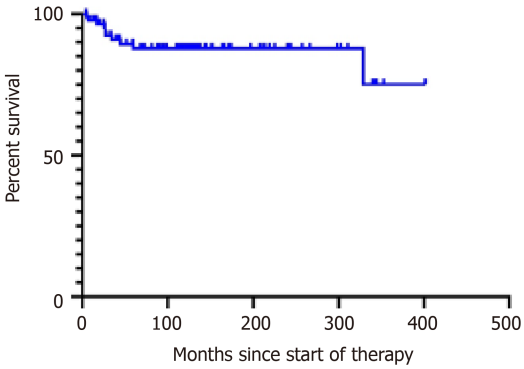


Figure 1 Overall survival of 88 patients with Hodgkin's lymphoma.

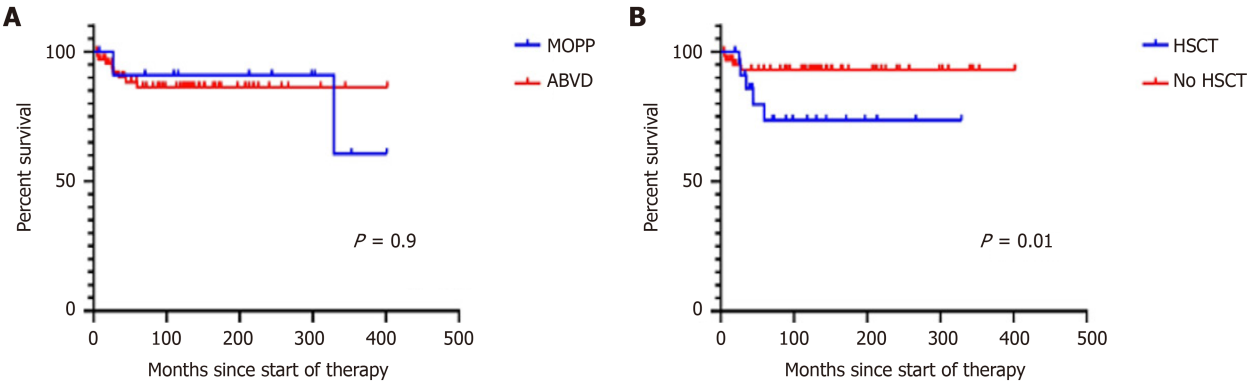


Figure 2 Overall survival of 88 patients with Hodgkin's lymphoma treated with either MOPP or ABVD (A) and treated either with or without hematopoietic stem cell transplants (B). ABVD: adriamycin bleomycin vinblastine and dacarbazine; HSCT: Hematopoietic stem cell transplant; MOPP: mechlorethamine, Oncovin, procarbazine, and prednisone.

Radiation therapy is included in treatment strategies. We have previously suggested that in Mexico, and probably in other underprivileged circumstances where RT facilities are suboptimal, patients with early stages of HL should be treated with ChT alone. The message being "conventional ChT is better than a poor RT"[19,21-24]. The results that we present here support the previous observations. Additionally, patients with relapsed/refractory disease were successfully rescued with ICE followed by HSCT. Figure 3 shows the OS of the patients classified by the prognostic score described by Hayden *et al*[24].

The main observations of treatment which we present here are (1) ABVD was offered to all patients regardless of their clinical stage. (2) Four cycles were administered to patients with stages I and II and six cycles to those with stages III and IV. (3) Two additional cycles were given to all patients after a negative CT scan after receiving 4-6 cycles. (4) No interim FDG-PET scans were done, reserving them for the

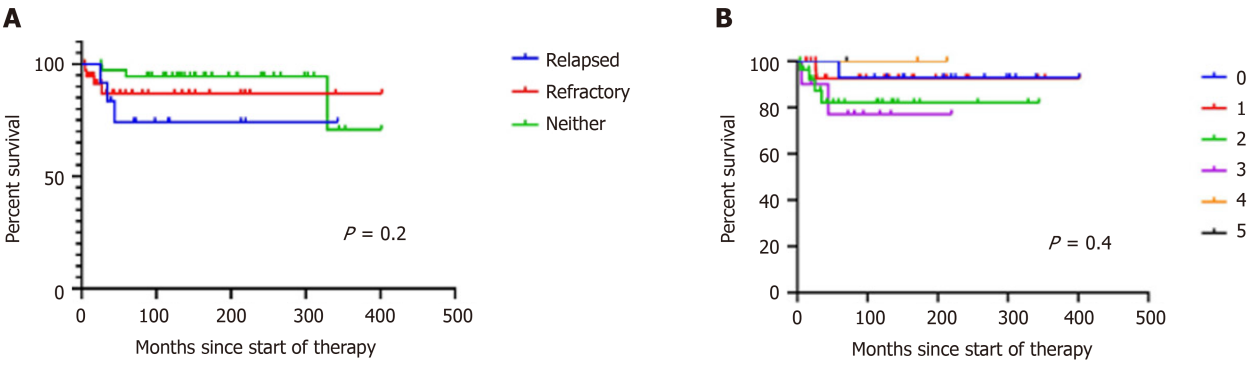


Figure 3 Overall survival of 88 patients with Hodgkin's lymphoma with or without relapsed or refractory disease (A), and overall survival of the patients with Hodgkin's lymphoma classified as described by Diefenbach *et al* (B).

end of treatment. And (5) Relapsed or refractory patients were given ICE followed by an HSCT. The limitations of this study include a relatively small and heterogeneous sample, the potential of referral bias, a high proportion of patients lost to follow-up, and socioeconomic factors.

This simplified approach to the treatment of patients with HL demonstrates adequate results with an OS of 88% at 26 years regardless of the clinical stage or relapsed/refractory disease. Additional data are needed to confirm the observations, which may be useful in circumstances of a restrained economy. The use of novel and expensive drugs such as pembrolizumab, nivolumab, panabinostat, idelalisib, mocetinostat, brentuximab vedotin and others[4,24] should be reserved for multi-relapsed cases and does not appear to be essential as frontline therapy.

CONCLUSION

HL may be less aggressive in the Mexican than in Caucasian populations. Combined chemotherapy without radiotherapy achieves acceptable results. In our context with healthcare limited-resources, chemotherapy alone with ABVD continues to be the treatment of choice in patients with HL.

ARTICLE HIGHLIGHTS

Research background

Hodgkin´s lymphoma (HL) can be treated with different alternatives, the performance of newer and older chemotherapy schemes are unknown in some circumstances.

Research motivation

The motivation was to describe the performance of treatment of HL in a middle-income country.

Research objectives

The objective was to determine performance of classic therapies for HL.

Research methods

This was a comparative study of therapies for HL in a single center over a long-term period.

Research results

HL may be less aggressive in the Mexican population. In addition, the classical ABVD regimen achieved long-term survival in a significant proportion of patients.

Research conclusions

Combined chemotherapy has acceptable efficacy in patients with HL. Our results suggest that classical treatment schemes continue to be an effective alternative. More

studies should be conducted.

Research perspectives

Classic therapies for HL may still be preferable over novel therapies in middle-income countries. The use of ABVD combined with other immunomodulatory agents could be a potential solution for patients not experiencing favorable outcomes.

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ORIGINAL ARTICLE

Retrospective Study

Role of mammogram and ultrasound imaging in predicting breast cancer subtypes in screening and symptomatic patients

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Abstract

BACKGROUND

Breast cancer (BC) radiogenomics, or correlation analysis of imaging features and BC molecular subtypes, can complement genetic analysis with less resource-intensive diagnostic methods to provide an early and accurate triage of BC. This is pertinent because BC is the most prevalent cancer amongst adult women, resulting in rising demands on public health resources.

AIM

To find combinations of mammogram and ultrasound imaging features that predict BC molecular subtypes in a sample of screening and symptomatic patients.

METHODS

This retrospective study evaluated 328 consecutive patients in 2017-2018 with histologically confirmed BC, of which 237 (72%) presented with symptoms and 91 (28%) were detected *via* a screening program. All the patients underwent mammography and ultrasound imaging prior to biopsy. The images were retrospectively read by two breast-imaging radiologists with 5-10 years of experience with no knowledge of the histology results to ensure statistical independence. To test the hypothesis that imaging features are correlated with tumor subtypes, univariate binomial and multinomial logistic regression models were performed. Our study also used the multivariate logistic regression (with and without interaction terms) to identify combinations of mammogram and ultrasound (US) imaging characteristics predictive of molecular subtypes.

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RESULTS

The presence of circumscribed margins, posterior enhancement, and large size is correlated with triple-negative BC (TNBC), while high-risk microcalcifications and microlobulated margins is predictive of HER2-enriched cancers. Ductal carcinoma *in situ* is characterized by small size on ultrasound, absence of posterior acoustic features, and architectural distortion on mammogram, while luminal subtypes tend to be small, with spiculated margins and posterior acoustic shadowing (Luminal A type). These results are broadly consistent with findings from prior studies. In addition, we also find that US size signals a higher odds ratio for TNBC if presented during screening. As TNBC tends to display sonographic features such as circumscribed margins and posterior enhancement, resulting in visual similarity with benign common lesions, at the screening stage, size may be a useful factor in deciding whether to recommend a biopsy.

CONCLUSION

Several imaging features were shown to be independent variables predicting molecular subtypes of BC. Knowledge of such correlations could help clinicians stratify BC patients, possibly enabling earlier treatment or aiding in therapeutic decisions in countries where receptor testing is not readily available.

Key Words: Hormone receptor; Molecular subtype; Ultrasonography; Mammography; Triple-negative cancer; Breast cancer screening

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Core Tip: Ultrasound and mammogram imaging features are correlated with breast cancer (BC) molecular subtypes. Knowledge of such correlations helps clinicians stratify patients, enabling earlier treatment or aiding therapeutic decisions. In a sample of symptomatic and asymptomatic (screening) patients, multivariate logistic regression showed that a combination of imaging features can distinguish: (1) Hormone receptor positive *vs* hormone receptor negative; (2) Triple negative BC (TNBC) *vs* non-TNBC; and (3) HER2+ (human epidermal receptor positive) *vs* non-HER2+ BC.

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INTRODUCTION

Breast cancer (BC) radiogenomics has been a prolific field of research in recent years, with an expanding number of studies examining the extent to which imaging can be utilized as a screening adjunct in the preliminary diagnosis of BC molecular subtypes [1]. While magnetic resonance imaging (MRI) has been the mainstay of radiogenomics, as it can produce a wide range of imaging features, a few studies have also utilized readily available modalities, such as mammogram (MG) and hand-guided ultrasound (US). These highlight the advantages of using cost-effective imaging tools that can be used for an early and accurate diagnosis of the cancer subtype[2].

The use of non-invasive, less resource-intensive methods to predict BC subtypes has practical significance, for two reasons. First, as BC is the most common cancer amongst adult women in the world, leveraging on radiologic imaging as a proxy for expensive genetic tests may help to lower the public health burden, particularly in the context of less developed countries where detailed and costly histopathologic analysis is not readily available. Second, BC is a heterogeneous disease—receptor expression and gene amplification profiles have different prognoses for disease progression and therapeutic response. Thus, radiologic imaging as an adjunct to genetic profiling can assist in pre-treatment planning and provide an additional level of analysis for radio-pathologic correlation discussions.

Prior research has established some degree of consensus regarding the correlation of imaging features with BC subtype, with ultrasound margins, posterior acoustic features, and high-risk microcalcifications (on MG) found to be independent imaging differentiators between molecular subtypes[3].

While previous studies focused mainly on symptomatic patients, this study aims to analyze which combination of US and MG imaging features are the most predictive of molecular subtypes in a patient population that comprises both symptomatic and asymptomatic (breast screening) patients. A unique feature of our dataset is a substantial proportion—slightly under 30% of the patients in our sample patients had cancers detected *via* our national screening program (91 of 328, 28%). This allows us to examine if imaging features indicative of higher-grade cancers can be detected in asymptomatic patients who undergo routine screening checks. In addition, due to the inclusion of screening patients, a significant proportion of cancers detected in our sample included non-invasive ductal carcinoma *in situ* (DCIS) (or 53 of 328, 16%). Thus, we were also able to analyze its mammographic and sonographic characteristics in relation to the four subtypes of invasive BCs, which to the best of our knowledge, has not been published in the existing literature.

MATERIALS AND METHODS

Patient data and selection criteria

Approval for this study was obtained from the local institutional review board. The need for informed consent for this study was waived due to the retrospective design, as patients had given permission to be included in the hospital's BC registry and the use of their unidentified data for research.

To minimize selection bias, our sample included consecutive patients with histologically confirmed BC diagnosed from January 2017 to December 2018. The surgical notes, histology reports, and medical images of 328 patients were retrieved *via* the hospital's electronic medical record system. The images were retrospectively read by two breast-imaging radiologists with 5-10 years of experience with no knowledge of the histology results to ensure statistical independence with regards to the dependent variable(s).

Imaging equipment and assessment

Standard two-view digital mammography was performed, with additional views when necessary, using Fuji or GE mammography units. Mammograms were interpreted by two breast imaging radiologists who were blinded to the histopathology report. Following the American College of Radiology (ACR) Breast Imaging Reporting and Data System (BI-RADS) reporting lexicon, abnormal mammographic findings were classified as an asymmetry, mass, architectural distortion, or suspicious microcalcifications.

Breast ultrasound was performed by ultrasound technologists using 12-5 Mhz transducers, with images interpreted by two breast imaging radiologists who were blinded to the histopathology report. Final decisions were made with consensus agreement among the radiologists. Each tumor was measured in three planes, with color Doppler images also acquired. Following the ACR BI-RADS guidelines, the lesion's margins, echogenicity, posterior features, and vascularity (Adler's index) were documented, along with size on ultrasound (Table 1).

Immunochemistry and classification of molecular subtypes

The expression status of the ER, PR, and HER2 receptors was assessed using an avidin-biotin complex immunohistochemical technique. The ER/PR statuses were evaluated using the Allred score based on the proportion of positively stained nuclei. Allred scores of at least 3 were considered as hormone receptor positive (ER+/PR+). The HER2 staining intensity was scored from 0 to 3+. Scores of 3+ were classified as HER2 positive (HER2+), whereas scores of 0/1+ were considered as HER2 negative (HER2-). Tumors with scores of 2+ were further assessed with fluorescence *in situ* hybridization to determine their HER2 status. The threshold ratio of the HER2 gene signal to the chromosome 17 probe signal was 2.2, above which the tumor was classified as HER2+ and below which the tumor was classified as HER2-.

For this paper, we have classified Luminal A tumors as being ER+/PR+ and HER2-, Luminal B tumors as being ER+/PR+ and HER2+, HER2-enriched as ER-/PR- and HER2+, and triple negative BC (TNBC) as being ER-/PR- and HER2-.

Table 1 Classification of imaging features based on American College of Radiology Breast Imaging Reporting and Data System Lexicon

Imaging feature	Classification
Mammogram	
Appearance	Mass
	Asymmetry
	High risk microcalcifications[1]
	Architectural distortion
Ultrasound	
Margins	Spiculated
	Microlobulated
	Circumscribed
Posterior acoustic features	Shadowing
	Enhancement
	Mixed
	None
Size	Maximum dimension on ultrasound (in mm)
Echogenicity	Homogenous
	Heterogeneous
	Complex cystic
Adler's index	Low (Grade I)
	Medium (Grade II)
	High (Grade III)

[1]High risk microcalcifications refer to microcalcifications which show grouped, linear, or segmental distribution, or with a pleomorphic or branching morphology.

Statistical analysis

Descriptive statistics were generated for the 328 patients included in the study. Clinicopathologic, mammogram, and ultrasound characteristics were based on the ACR BI-RADS lexicon. Data was summarized using frequency and percentage for qualitative variables, mean ± SD, and range for quantitative variables, as listed in Table 2.

To test the primary hypothesis that imaging features are correlated with tumor subtypes, univariate binomial and multinomial logistic regression models were performed. Our study also used the multivariate logistic regression (with and without interaction terms), to assess if a joint combination of MG and US imaging characteristics is predictive of molecular subtypes, and the results are summarized in Tables 3-6. The specification for the multivariate model was derived using stepwise regression (SLE = 0.05, SLS = 0.05). Discrimination and classification of the final multivariate models or predictor were assessed using the area under the receiver operating characteristic curve (AUC). All analysis was performed with the use of statistical software (RStudio, Version 1.3.1073), using the mlogit (v.1.1-0; Croissant, 2002), nnet (v2.13.1; Venables & Ripley, 2002)[4,5], and pROC (Robin *et al*[6]) packages.

RESULTS

Out of the 328 cases, 139 (48%) were ER+/PR+, HER2- (Luminal A type); 38 (12%) were ER+/PR+, HER2+ (Luminal B type); 50 (15%) were ER-/PR-, HER2+ (HER2-enriched type); 48 (15%) were ER-/PR-, HER2- (triple negative type), and 53 (16%) were DCIS. The age range of the study sample was 31-86-years-old, with a mean age of 61.1 years. Across the four molecular subtypes, there was no significant difference in age. Ninety-one (27%) cases were detected *via* breast screening, while the remaining

Table 2 Distribution of demographic and imaging parameters based on molecular subtype

	Total (*n* = 328)	DCIS (*n* = 53)	Luminal A (*n* = 139)	Luminal B (*n* = 38)	Her2 enriched (*n* = 50)	Triple negative (*n* = 48)
Mean age	61.1 ± 11.75	59.1 ± 11.67	61.8 ± 11.80	60.2 ± 12.23	62.0 ± 9.63	61.3 ± 12.60
Presentation						
Clinic	237 (72%)	29 (55%)	98 (71%)	28 (74%)	36 (72%)	46 (96%)
Screening	91 (28%)	24 (45%)	41 (29%)	10 (26%)	14 (28%)	2 (4%)
Mass	200 (61%)	14 (26%)	91 (65%)	29 (76%)	29 (58%)	37 (77%)
Architectural distortion	17 (6%)	7 (13%)	10 (7%)	0 (0%)	0 (0%)	0 (0%)
Asymmetry	40 (12%)	6 (11%)	16 (12%)	3 (8%)	9 (18%)	6 (13%)
High-risk microcalcifications	105 (32%)	27 (51%)	33 (24%)	12 (32%)	24 (48%)	9 (19%)
Tumor size (on USG)						
< 20	136 (41%)	22 (42%)	71 (51%)	20 (53%)	19 (38%)	4 (8%)
≥ 20	154 (47%)	9 (17%)	55 (40%)	17 (45%)	29 (58%)	44 (92%)
Margins (on USG)						
Spiculated	100 (30%)	5 (9%)	63 (45%)	12 (32%)	8 (16%)	8 (17%)
Microlobulated	174 (60%)	23 (43%)	59 (42%)	25 (66%)	40 (80%)	29 (60%)
Circumscribed	16 (5%)	3 (6%)	4 (3%)	0 (0%)	0 (0%)	11 (23%)
Echogenicity (on USG)						
Heterogeneous	144 (44%)	13 (25%)	58 (42%)	19 (50%)	28 (56%)	26 (54%)
Homogenous	146 (45%)	18 (34%)	68 (49%)	18 (47%)	20 (40%)	22 (46%)
Posterior acoustic features						
Shadow	74 (23%)	5 (9%)	49 (35%)	10 (26%)	4 (8%)	6 (13%)
Enhancement	85 (26%)	5 (9%)	23 (17%)	8 (21%)	6 (32%)	33 (69%)
Mixed	36 (11%)	5 (9%)	15 (11%)	3 (8%)	9 (18%)	4 (8%)
None	95 (29%)	16 (30%)	39 (28%)	16 (42%)	19 (38%)	5 (10%)
Adler's vascularity						
High (Grade II & III)	81 (25%)	7 (13%)	22 (16%)	11 (29%)	21 (42%)	20 (42%)
Low (Grade I)	209 (64%)	24 (45%)	104 (75%)	26 (68%)	27 (54%)	28 (58%)
Axillary nodes						
Present	56 (17%)	0 (0%)	25 (18%)	1 (3%)	14 (28%)	16 (33%)
Absent	234 (71%)	53 (100%)	101 (73%)	36 (95%)	34 (68%)	32 (67%)
Not visible on US	38 (14%)	22 (42%)	13 (9%)	1 (3%)	2 (1%)	0 (0%)

DCIS: Ductal carcinoma *in situ*; US: Ultrasound; USG: Ultrasonography.

191 (69%) cases presented with symptoms at the breast clinic. Most of the cases (89%) detected *via* breast screening were classified as DCIS or hormone receptor positive cancer, and only a minority (11%) of cases detected *via* screening were hormone receptor negative cancers.

Microcalcifications

High risk microcalcifications were found in slightly under one third of the full sample (n = 105, 32%) but were more prevalent amongst BC patients with the subtype HER2-enriched (n = 24, 48%) and DCIS (n = 27, 51%) (Figure 1A). In the univariate multinomial logistic regression (with reference to Luminal A as the baseline), the presence of high risk microcalcifications on mammography was positively associated

Table 3 Binomial univariate and multivariate logistic regressions (ER/PR positive *vs* negative)

ER/PR positive *vs* negative		Univariate			Multivariate (AUC = 0.792)		
		P	OR	CI	*P*	OR	CI
Posterior acoustic features[1]	Enhancement	[d]	0.46	(0.193 1.050)	[d]	0.45	(0.174 1.143)
	Shadowing	[b]	4.26	(1.617 11.633)	Not significant		
Margins[2]	Spiculated	[a]	4.16	(2.268 7.975)	[a]	0.41	(1.085 4.606)
	Circumscribed	[a]	0.15	(0.023 0.594)	Not significant		
Size	Small < 20 mm	[c]	4.51	(2.499 7.443)	[a]	2.74	(1.475 5.204)

[1]Mixed posterior acoustic features were set as the baseline with OR = 1.
[2]Microlobulated ultrasound margins were set as the baseline with OR = 1.
[a]Significant at the 0.001 level.
[b]Significant at the 0.01 level.
[c]Significant at the 0.05 level.
[d]Significant at the 0.10 level.
Area under the receiver operating characteristic curve for multivariate regression on posterior acoustic features, ultrasound margins, MM high-risk microcalcifications, and size (large or small).

Table 4 Binomial univariate and multivariate logistic regressions (Triple-negative breast cancer *vs* Non-triple-negative breast cancer)

TNBC *vs* Non-TNBC		Univariate			Multivariate (AUC = 0.853)		
		P	OR	CI	*P*	OR	CI
Posterior acoustic features[1]	Enhancement	[b]	5.08	(1.808 18.201)	[a]	4.77	(1.556 18.291)
Margins[2]	Spiculated	[a]	0.43	(0.179 0.951)	Not significant		
	Circumscribed	[c]	11.00	(3.712 37.166)	[b]	8.24	(2.151 38.923)
Size	Large > 20 mm	[c]	13.2	(5.152 44.845)	[c]	10.5	(3.792 38.436)
Axillary node metastasis	Yes	[b]	2.52	(1.247 4.988)	Not significant		
Adler's Index	High	[a]	2.12	(1.104 4.020)	Not significant		
[a]Screening	Yes	[d]	0.003	(0.000 0.222)	[d]	0.004	(0.000 0.452)
[a]Interaction term	Screen × unit size	[d]	1.17	(1.003 1.505)	[d]	1.16	(1.001 1.430)

[1]Mixed posterior acoustic features were set as the baseline with OR = 1.
[2]Microlobulated ultrasound margins were set as the baseline with OR = 1.
[a]Significant at the 0.001 level.
[b]Significant at the 0.01 level.
[c]Significant at the 0.05 level.
[d]Significant at the 0.10 level.
Area under the receiver operating characteristic curve for multivariate regression on posterior acoustic features, ultrasound margins, and size (large or small). AUC: Area under the receiver operating characteristic curve; TNBC: Triple-negative breast cancer.

with the presence of HER2 receptor expression (P = 0.002, OR 2.97) and DCIS ($P \leq$ 0.001, OR 3.34). In the multivariate logistic regression, high-risk microcalcifications also increase the relative odds of the tumor being a HER2-enriched type ($P \leq$ 0.001, OR 3.38) (Table 5).

Architectural distortions

Architectural distortions presented rarely in DCIS (n = 7, 13%) and Luminal A subtypes (n = 10, 7%), and were not common in the other three subtypes of BC. On univariate analysis, architectural distortions were significantly associated with DCIS (P = 0.024, OR 3.23) (Table 6), but no statistically significant relationship with Luminal A type was found.

Margins

The vast majority of tumors in our study (n = 274, 94%) had non-circumscribed

Table 5 Binomial univariate and multivariate logistic regressions (HER2+ *vs* Non HER2+)

HER2+ *vs* Non HER2+		Univariate			Multivariate (AUC = 0.747)		
		P	OR	CI	*P*	OR	CI
Margins[1]	Microlobulated	[c]	3.92	(1.830 9.410)	[b]	3.26	(1.469 8.026)
	Circumscribed	Not significant			Not significant		
High-risk microcalcifications	Present	[c]	3.51	(1.804 6.821)	[c]	3.38	(1.685 6.816)
Adler's Index	High	[a]	2.32	(1.203 4.437)	[a]	1.99	(0.999 4.010)

[1]Spiculated ultrasound margins were set as the baseline with OR = 1.
[a]Significant at the 0.001 level.
[b]Significant at the 0.01 level.
[c]Significant at the 0.05 level.
[d]Significant at the 0.10 level.
Area under the receiver operating characteristic curve for multivariate regression on ultrasound (US) margins, MM high-risk microcalcifications, and US Adler index (low or intermediate/high). AUC: Area under the receiver operating characteristic curve.

Table 6 Binomial univariate and multivariate logistic regressions (Ductal carcinoma *in situ* *vs* Invasive cancers)

DCIS *vs* Invasive cancers		Univariate			Multivariate (AUC = 0.719)		
		P	OR	CI	*P*	OR	CI
Posterior acoustic features[1]	None	[a]	3.24	(1.204 10.292)	[a]	3.45	(1.255 11.118)
High-risk microcalcifications	Present	[b]	2.80	(1.516 5.208)	Not significant		
Architectural distortions	Yes	[b]	5.34	(1.546 16.709)	Not significant		
Size	Small < 20 mm	[a]	2.59	(1.420 7.361)	[a]	2.72	(1.172 6.182)

[1]Posterior acoustic enhancement was set as the baseline with OR = 1.
[a]Significant at the 0.001 level.
[b]Significant at the 0.01 level.
[c]Significant at the 0.05 level.
[d]Significant at the 0.10 level.
Area under the receiver operating characteristic curve for multivariate regression on posterior acoustic features, MM high-risk microcalcifications, architectural distortions, and size (large or small). AUC: Area under the receiver operating characteristic curve; DCIS: Ductal carcinoma *in situ*.

margins, of which more than half were classified as microlobulated (n = 174, 60%), followed by spiculated margins (n = 100, 34%). Only 6% of all lesions showed circumscribed margins, of which the majority were TNBC cases (n = 11, 4%), and the rest were DCIS (n = 3, 1%). Based on the univariate and multivariate logistic regressions, the presence of circumscribed margins was found to significantly increase the relative odds of TNBC cancer (univariate: P = 0.003, OR 11.0; multivariate: P = 0.004, OR 8.24) (Figure 2A, Table 4). Moreover, the presence of a spiculated margin on sonography was common in Luminal A cancers (n = 67, 53%), but rarely presented in TN and HER2+ tumors (n = 16, 16%). Thus, if the tumor shows spiculated margins, the odds of it being a hormone receptor positive subtype is higher, as confirmed in the univariate and multivariate regressions (univariate: P = 0.000, OR 4.16; multivariate: P = 0.03, OR 2.20) (Table 3).

Posterior acoustic features

Posterior acoustic enhancement was more common in HER2-enriched and TN tumors (n = 16, 32% and n = 33, 69%), compared to luminal cancers (n = 31, 19%) and DCIS (n = 5, 9%). It is a strong predictor for TNBC cancers *vs* non-TNBC cancers (univariate: P = 0.005 OR 5.08; multivariate: P = 0.01 OR 4.77) (Figure 2B, Table 4). In the multinomial logistic regressions, posterior enhancement signaled higher relative odds of the tumor being a TNBC cancer relative to Luminal A, DCIS, and HER2-enriched tumors (Table 4).

Conversely, posterior acoustic shadowing is associated with slightly higher odds of the tumor being a hormone positive type. In Luminal A cancers, posterior shadowing (n = 49, 39%) was more prevalent compared to the other three subtypes of invasive

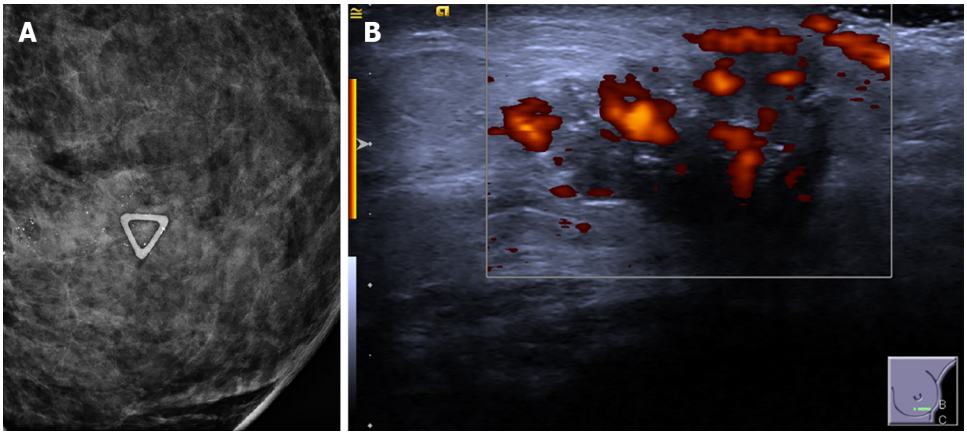


Figure 1 HER2-enriched invasive cancer. A: High-risk microcalcifications on mammogram of the left breast are seen within the palpable mass (denoted by triangular skin marker); B: Irregular hypoechoic mass showing increased internal vascularity (Adler Index Grade III) and internal echogenic foci representing microcalcifications.

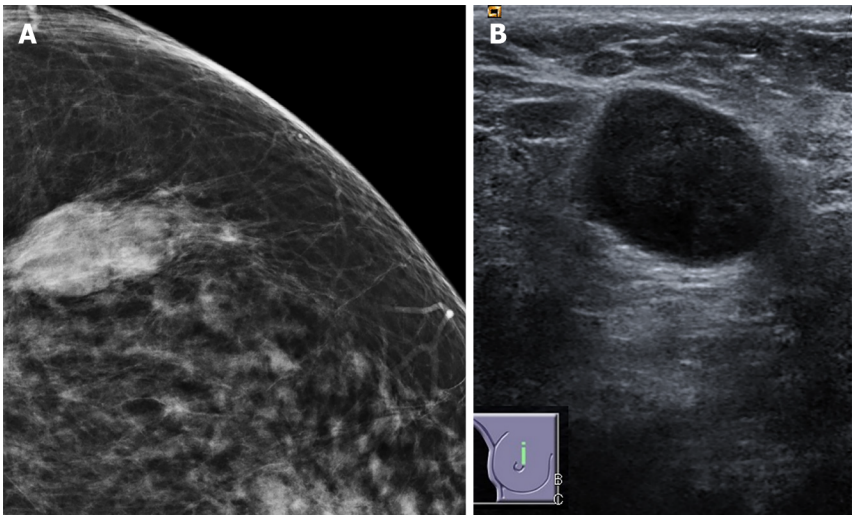


Figure 2 Triple negative breast cancer. A: Circumscribed mass on mammogram; B: Circumscribed hypoechoic mass with posterior acoustic enhancement on ultrasound.

cancer (n = 20, 15%). This was confirmed in the univariate binomial and multinomial regressions, posterior acoustic shadowing suggested a higher likelihood of the tumor being a hormone receptor positive type (univariate: P = 0.037, OR 4.26) (Table 3). However, there was no statistically significant positive association in the multivariate model specifications, possibly due to the lack of statistical power given the limited sample size.

Of the DCIS tumors that were visible on US (60% of all DCIS), the majority did not show any posterior acoustic features (n = 16, 51%). In comparison, most of the invasive cancers showed either posterior shadowing, enhancement, or a mix of both and only a minority showed no posterior acoustic features (n = 79, 29%). Absence of posterior acoustic features favors DCIS in the univariate and multivariate regressions (univariate: P = 0.028, OR 3.24; multivariate: P = 0.090, OR 2.49) (Table 6).

Vascularity

Tumors with a high score on the Adler index (II or III) comprised a minority of the sample (n = 55, 23%), and were more predominant in the HER2 enriched and TNBC subtypes (n = 21, 42%, n = 20, 42%, respectively) (Figure 1B). High vascularity was statistically significant as a predictor of HER2-enriched status in both the multivariate and univariate regressions (univariate regression: P = 0.011, OR 2.32; multivariate regression: P = 0.05, OR 1.99) (Table 5), while low vascularity is more likely in Luminal A tumors, corroborating similar findings in other studies[3] (Table 3). Similar to another study which showed a positive association between TNBC and high

vascularity using optoacoustic US imaging[7], we also found a positive association between TNBC cancers and vascularity in the univariate, but not multivariate specifications (univariate: $P = 0.022$, OR 2.12) (Table 4).

Tumor size

Tumor size was one of the strongest predictors of TNBC. If the lesion is large (> 20 mm), the relative odds of the tumor being a TNBC subtype would be 13 times higher compared to a non-TNBC type (univariate regression: $P \leq 0.001$, OR 13.20; Multivariate regression: $P \leq 0.001$, OR 10.54) (Table 4). In the multinomial regressions, large tumor size consistently predicted a higher likelihood of the tumor being TNBC *vs* all other subtypes. The high positive correlation between size and TNBC status reflects the fact that almost all the TNBC tumors were large ($n = 44$, 92%), compared to around 40% of the Luminal A/B tumors, and 60% of the HER2 enriched tumors. Large tumor size also signaled a higher likelihood of HER2-enriched status *vs* DCIS and Luminal A in the multinomial regression ($P = 0.008$, OR 3.73; $P = 0.05$, OR 1.97) (Table 7).

In contrast, sonographically visible DCIS and hormone receptor positive cancers are typically small (< 20 mm). In the univariate and multivariate regressions, small size increases the relative likelihood of the tumor being a DCIS type by 2.5-3 times, compared to invasive cancers (univariate: $P = 0.006$, OR 2.59; multivariate: $P = 0.012$, OR 3.02) (Table 6). Within the invasive cancer subtypes, a small sized tumor signals a higher likelihood of the tumor being either Luminal A/B (hormone receptor positive), compared to HER2-enriched or TNBC (univariate: $P \leq 0.001$, OR 4.26; multivariate: $P = 0.002$, OR 2.74) (Table 3).

Axillary node metastasis

Axillary node metastasis was most common in TNBC cancers ($n = 16$, 33%), followed by Luminal A ($n = 25$, 18%), and least prevalent in the Luminal B subtype ($n = 1$, 3%). We found evidence that TNBC is associated with a higher positive rate of axillary adenopathy compared to the non-TNBC tumors in the univariate binomial logistic regression ($P = 0.008$, OR 2.53) (TNBC *vs* non TNBC) but not the multivariate binomial logistic regression (Table 4). In the univariate multinomial model, the presence of axillary adenopathy represented a higher likelihood of the tumor being TNBC ($P = 0.029$, OR 18.50) or Luminal A subtype ($P = 0.043$, OR 3.08) relative to Luminal B subtype (Table 4). Our results are in keeping with several studies who also recorded a positive association between the presence of axillary node metastases and TNBC cancers[8,9]. However, the findings on axillary adenopathy and TNBC association have been mixed in the literature, with some other studies finding a negative association instead[10].

Multivariate logistic regressions

Although the univariate regressions show that a few key radiologic imaging features are statistically significant as independent prognostic indicators of the BC subtype, using a joint combination of imaging features could increase the reliability of the preliminary diagnosis. This is because US image acquisition is highly user dependent, and radiologic interpretation may sometimes be equivocal, for instance, in the case of lobulated (microlobulated) *vs* angular (spiculated) margins.

Similar to previous studies[7,11], we estimated multivariate binomial logistic regressions using the stepwise regression approach (SLE = 0.05, SLS = 0.05), with the final model specification determined by the Akaike information criterion. We catalogue four distinct categorizations: (1) Hormone-receptor positive *vs* hormone-receptive negative invasive cancers; (2) TNBC *vs* non-TNBC invasive cancers; (3) HER2-enriched *vs* non HER2-enriched invasive cancers; and (4) DCIS *vs* invasive cancers. The distinguishing characteristics for each category are: (1) A small lesion (< 20 mm), with spiculated margins, the absence of posterior acoustic enhancement and absence of high-risk microcalcifications, is more likely associated with hormone-receptor positive status (Figure 3); (2) Posterior acoustic enhancement, circumscribed margins, and large tumor size was predictive of TNBC status; (3) Microlobulated margins, high-risk microcalcifications, and high vascularity was predictive of HER2-enriched status; and (4) Absence of posterior acoustic features and small size on ultrasound was associated with DCIS compared to invasive cancers. A receiver operating characteristic curve was plotted using the pROC package in R; the performance of the multivariate models based on the AUC was (1) 0.792; (2) 0.853; (3) 0.747; and (4) 0.719, respectively.

Table 7 Multinomial univariate logistic regressions: Impact of imaging features on relative odds of molecular subtypes

			Luminal A baseline			Luminal B baseline			DCIS baseline			HER2 enriched baseline			TNBC baseline		
			P value	OR	95%CI	*P* value	OR	95%CI	*P* value	OR	95%CI	*P* value	OR	95%CI	*P* value	OR	95%CI
Posterior acoustic features	Shadowing	Luminal A *vs*							[d]	**3.27**	**(0.832 12.83)**	[b]	**7.35**	**(1.979 27.300)**			
		Luminal B *vs*										[a]	**7.50**	**(1.307 43.030)**			
		DCIS *vs*	[d]	0.31	(0.078 1.202)												
		HER2+ *vs*	[b]	0.14	(0.037 0.505)	[a]	0.13	(0.023 0.765)									
		TNBC *vs*															
	Enhancement	Luminal A *vs*													[b]	0.19	(0.055 0.633)
		Luminal B *vs*															
		DCIS *vs*													[a]	0.12	(0.024 0.610)
		HER2+ *vs*													[a]	0.22	(0.058 0.807)
		TNBC *vs*	[b]	**5.38**	**(1.581 18.310)**				[a]	**8.25**	**(1.638 41.55)**	[a]	**4.64**	**(1.239 17.38)**			
Margins (on US)	Spiculated	Luminal A *vs*				[a]	**2.45**	**(1.130 5.31)**	[b]	**5.41**	**(1.931 15.14)**	[c]	**5.88**	**(2.544 13.58)**	[c]	**4.26**	**(1.805 10.056)**
		Luminal B *vs*	[a]	0.41	(0.188 0.885)							[d]	**2.40**	**(0.861 6.690)**			
		DCIS *vs*	[b]	0.19	(0.066 0.518)												
		HER2+ *vs*	[c]	0.17	(0.074 0.393)	[d]	0.42	(0.150 1.16)									
		TNBC *vs*	[c]	0.24	(0.099 0.554)												
	Circumscribed	Luminal A *vs*													[b]	0.09	(0.019 0.445)
		Luminal B *vs*															
		DCIS *vs*															
		HER2+ *vs*															
		TNBC *vs*	[b]	**10.8**	**(2.246 52.043)**												
Size	Large	Luminal A *vs*										[a]	0.51	(0.258 0.999)	[c]	0.07	(0.024 0.208)
		Luminal B *vs*													[c]	0.08	(0.023 0.259)
		DCIS *vs*													[c]	0.04	(0.010 0.134)
		HER2+ *vs*	[a]	**1.97**	**(1.001 3.878)**										[c]	0.14	(0.043 0.450)
		TNBC *vs*	[c]	**14.20**	**(4.811 41.915)**	[c]	**12.94**	**(3.856 43.427)**	[c]	**26.89**	**(7.445 97.114)**	**c**	**7.21**	**(2.224 23.354)**			
High-risk	Present	Luminal							[c]	0.30	(0.154	[b]	0.34	(0.171			

Microcals		A *vs*								0.583)			0.665)			
		Luminal B *vs*						[d]	0.44	(0.186 1.061)						
		DCIS *vs*	[c]	**3.34**	**(1.715 6.488)**	[d]	**2.25**	**(0.942 5.37)**						[b]	**4.50**	**(1.606 9.96)**
		HER2+ *vs*	[b]	**2.97**	**(1.504 5.844)**									[b]	**4.00**	**(1.824 11.10)**
		TNBC *vs*						[b]	0.22	(0.090 0.548)		0.25	(0.100 0.623)			
Adler	High	Luminal A *vs*									[c]	0.27	(0.131 0.566)	[b]	0.30	(0.142 0.618)
		Luminal B *vs*														
		DCIS *vs*									[d]	0.38	(0.136 1.037)	[d]	0.41	(0.147 1.131)
		HER2+ *vs*	[c]	**3.68**	**(1.767 7.650)**			[d]	**2.67**	**(0.965 7.37)**						
		TNBC *vs*	[b]	**3.38**	**(1.618 7.045)**			[d]	**2.45**	**(0.884 6.78)**						
Axillary node adenopathy	Yes	Luminal A *vs*				[a]	**3.08**	**(1.063 61.96)**						[a]	0.44	(0.209 0.919)
		Luminal B *vs*	[a]	0.12	(0.224 0.470)						[a]	0.07	(0.009 0.556)	[b]	0.05	(0.007 0.430)
		DCIS *vs*														
		HER2+ *vs*				[a]	**14.40**	**(1.798 115.17)**								
		TNBC *vs*	[a]	**2.28**	**(1.088 4.778)**	[b]	**18.50**	**(2.323 147.34)**								

[a]Significant at the 0.001 level.
[b]Significant at the 0.01 level.
[c]Significant at the 0.05 level.
[d]Significant at the 0.10 level.
Only statistically significant results are shown. Bold results show higher relative odds. DCIS: Ductal carcinoma *in situ*; TNBC: Triple-negative breast cancer; US: Ultrasound.

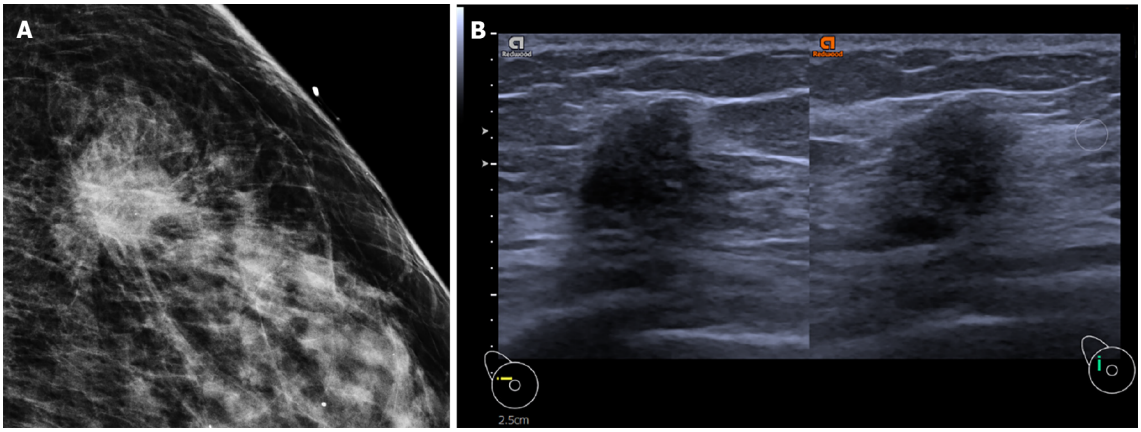


Figure 3 Luminal type invasive cancer. A: Spiculated mass on mammogram; B: Irregular hypoechoic mass with spiculated margin and posterior acoustic shadowing.

Screening and size of tumor

A significant proportion of the cancers in our sample were detected *via* routine breast screening (n = 91, 28%). Previous studies have shown that BCs detected *via* screening are typically smaller than symptomatic BCs, and that screening is an effective means of

detecting tumors at an earlier stage[12].

In our sample, screening detected tumors when they were smaller, even after controlling for molecular subtype. The mean size of hormone receptor negative tumors detected *via* screening was 19.0 mm *vs* 33.0 mm for hormone receptor negative cancers presenting with symptoms. The mean size of TNBC and HER2-enriched cancers that were detected *via* screening was 32.5 mm and 14.4 mm respectively *vs* 34.7 mm and 30.8 mm, respectively for symptomatic patients. However, as size is a less important predictor of disease severity than the biologic characteristics of the tumor based on its gene expression, a few prominent studies have downplayed the usefulness of breast screening in improving patient outcomes, considering its tendency for overdiagnosis of in-situ type cancers[13].

One of the main findings of our study is that TNBC cancers tend to have benign morphologic features, such as circumscribed margins and posterior enhancement. This raises possibility of mistaking it for common benign lesions such as cysts with echoes or fibroadenomas. The only unique differentiator is its positive correlation with size. Thus, in the screening context, size may be a useful factor in deciding whether to recommend an invasive biopsy or imaging follow-up for the lesion in question.

To estimate the impact of size on the probability of the tumor being a TNBC subtype conditional on the tumor presenting during screening, in the multivariate regressions, we specified an interaction term of screening with size, and find a marginally significant positive coefficient for the interaction term. Interpretively, this means that for every unit increase in lesion size, there are higher relative odds of the screening tumor being a TNBC subtype compared to non-TNBC, holding all other factors unchanged (P = 0.08, OR 1.18) (Table 4).

DISCUSSION

BC is a heterogenous disease, characterized by the varied imaging appearance, histologic and molecular profiles, and correspondingly different disease course across the various molecular subtypes. The different molecular types of BC have different biological behaviors at the cellular level, which influence the speed of invasion and destruction of the surrounding tissue, consequently affecting the macroscopic appearance of the tumor on mammogram and ultrasound.

Prior studies investigating the association of imaging features with molecular subtypes found evidence that cancers with posterior acoustic shadowing have higher odds of hormone-receptor positivity while those with posterior acoustic enhancement are likely to have negative receptor expression[14]. TNBC cancers were more likely to have circumscribed margins while hormone receptor positive cancers were more likely to show spiculated margins[15,16]. High risk microcalcifications detected on mammogram are associated with HER2-enriched cancers[17]. Our results are generally consistent with prior studies, with imaging features that correlate with the following subtypes: Spiculated margins were positively correlated with hormone receptor positive status (Luminal A or B). As hypothesized by earlier research, as luminal cancers tend to be lower grade and grow at a slower rate, they provoke a desmoplastic reaction, resulting in radiologic findings of spiculated margins. The desmoplastic reaction also affects acoustic impedance of the tumor to healthy tissue interface, causing excessive sonographic attenuation by the tumor, resulting in posterior shadowing[14,15,18].

High-risk microcalcifications, vascularity, and microlobulated margins, are positively associated with HER2 enriched cancers. Studies have shown that HER2 gene overexpression is linked to neo-angiogenesis *via* production of VEGF[19], while high-risk microcalcifications are due to the tendency of HER2 cancers to have a concomitant DCIS component[20]. Rapidly proliferating tumor cells, which consume the blood supply, lead to tumor necrosis and subsequent acidosis in the microenvironment, resulting in calcium accumulation in the ducts[21]. Our results concur with prior studies, which reported high risk microcalcifications to be more frequent in HER2+ tumors compared to luminal cancers[22].

A statistically significantly relationship exists between TNBC, which is the most aggressive molecular subtype, and posterior acoustic enhancement, circumscribed margins, and large tumor size. These are more cellular, grow rapidly, and do not generally incite a strong desmoplastic reaction from the surrounding healthy tissue[6]. The more regular interface between tumor and surrounding tissue probably results in a circumscribed margin, while internal necrosis and high cellularity probably attenuate the sound waves to a lesser degree, manifesting as posterior enhancement on

ultrasound. Concordant with other studies[8,11], US tumor size was a statistically significant predictor of TNBC cancers. In our sample, we found that if detected through breast screening (patients were asymptomatic), each unit increase in tumor size is associated with a higher odds ratio for TNBC cancer relative to the rest of the subtypes. This may imply that breast screening could be detecting TNBC at an earlier stage (smaller size, non-palpable), and that clinicians should prioritize biopsy of larger lumps when assessing screening populations.

DCIS is characterized by architectural distortions and high-risk microcalcifications on MG, but only 60% of DCIS tumors are visible on US (n = 31, 58%). High-risk microcalcifications tend to be absent in DCIS tumors that are sonographically visible. On US, a combination of small size and absence of posterior acoustic features favor DCIS. These two sonographic features are also commonly seen in benign lesions. However, similar to invasive cancers, the majority of sonographically visible DCIS show microlobulated or spiculated margins, whereas the overwhelming majority of benign lesions demonstrate circumscribed margins[23]. This highlights the importance of margin assessment of even small lesions on ultrasound, which depends on the skill of the performing technologist, as well as the use of a high-resolution probe.

In the current landscape of BC chemotherapeutic regimens, hormonal therapy is routinely used for hormone-receptor positive tumors (Luminal A/B), while targeted therapy, such as Herceptin, is available for cancers which overexpress HER2 (Luminal B/HER2-enriched). On the other hand, not only are TNBC tumors more aggressive (due to poor dedifferentiation), and have a higher recurrence rate, no targeted therapeutic strategy is currently available for the treatment of TNBC, leaving non-targeted chemotherapy as the only weapon in the chemotherapeutic arsenal. Stratification may allow for earlier resection times by aiding radiologists in deciding whether to biopsy or observe the lesion. As screening detects tumors at an earlier stage, large size is one discriminating feature that could point towards an aggressive TNBC subtype—prioritizing biopsy for these cases may benefit patient outcomes. In addition, histopathological analysis may not be readily available in certain developing countries, and an improved understanding of how the imaging features of BC correlate to molecular subtype would aid in tailoring the treatment strategy for patients who are cost constrained.

Our study had several limitations. First, as this was a retrospective study conducted at a single institution, it could have been subject to selection bias with respect to patients who arrive at our institution, even though we endeavored to minimize selection bias by including all consecutive patients within a fixed duration of time and blinding the assessing radiologists to the histology results. Second, due to the relatively small sample size, some of the subanalysis could have lacked statistical power to detect a significant difference in imaging features across molecular subtypes. Nevertheless, we see potential for further research, particularly in automated machine learning. From studies such as ours, imaging features identified as effective predictors could be used to customize deterministic algorithms for computer-extracted features, which could then be utilized at large-scale and with no inter-reader variability[2]. In fact, a recent study introduced a machine learning model for MRI classification of BC subtypes, guiding their radiomic feature selection and categorization by a review of the prior literature for imaging features that exhibited prognostic significance for various aspects of BC[24].

CONCLUSION

In conclusion, key features in mammographic and sonographic imaging were significantly associated with BC molecular subtypes. Knowledge of such correlations could help clinicians stratify BC patients according to their likely molecular subtype, potentially enabling earlier, more effective treatment or aiding in therapeutic decisions in countries where receptor testing is not readily available.

ARTICLE HIGHLIGHTS

Research background

There is evidence in the literature that breast cancer (BC) molecular subtypes often have characteristic imaging features on mammogram (MG), ultrasound (US) and magnetic resonance imaging. These imaging features on MG and US are of particular

interest as they are cost-effective and widely available even in many developing countries.

Research motivation

Thus far, research into the correlation between MG and US imaging features and BC subtypes has been based on populations of symptomatic patients, with the lack of data on an asymptomatic (screening) population highlighted as an area for future research. We wanted to thus use our data which consists of both screening and symptomatic patients to add to the body of knowledge on this issue. Also, our population includes patients with ductal carcinoma-in-situ (DCIS) which only a few papers have examined.

Research objectives

To correlate the MG and US imaging features with the molecular subtypes of BC (hormone receptor positive *vs* hormone receptor negative, triple-negative *vs* non-triple negative and HER2 positive *vs* HER2 negative) and DCIS in our population of screening and symptomatic patients.

Research methods

Our study is retrospective, with a population of 328 consecutive patients in 2017-18 with histologically confirmed BC. 237 (72%) were symptomatic, and 91 (28%) were detected *via* a screening program. All the patients underwent MG and US imaging prior to biopsy. The images were retrospectively interpreted by two breast-imaging radiologists with 5-10 years of experience who were blinded to the histology results to ensure statistical independence. To test the hypothesis that imaging features are correlated with tumor subtypes, univariate binomial and multinomial logistic regression models were performed. Also, multivariate logistic regression (with and without interaction terms) was utilized to identify combinations of MG and US imaging characteristics predictive of molecular subtypes.

Research results

Circumscribed margins, posterior enhancement, and large size are correlated with triple-negative BC (TNBC). High-risk microcalcifications and microlobulated margins is predictive of HER2-enriched cancers. DCIS is characterized by small size on US, absence of posterior acoustic features, and the presence of architectural distortion on MG. Hormone receptor positive subtypes tend to be small, with spiculated margins and posterior acoustic shadowing. These results are broadly consistent with findings from prior studies. In addition, we also find that US lesion size signals a higher odds ratio for TNBC if presented during screening.

Research conclusions

Several MG and US imaging features were shown to independently predict molecular subtypes of BC, in a population of both screening and symptomatic patients. Knowledge of such correlations could help clinicians stratify BC patients, possibly enabling earlier treatment for patients with triple negative cancer. This could also aid therapeutic decisions in countries where receptor testing is not readily available.

Research perspectives

To further research in this field, machine learning algorithms may be trained to recognize both the imaging characteristics as well as the radionomic characteristics of BC molecular subtypes, to see if this can further improve the predictive accuracy of imaging. More studies with asymptomatic populations of patients, and with differing ethnicities would also be useful to corroborate the results in our study.

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ORIGINAL ARTICLE

Retrospective Study

Features of primary pancreatic lymphoma: A bi-institutional review with an emphasis on typical and atypical imaging features

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Institutional review board statement: The study was reviewed and approved separately by the Institutional Review Boards of University of Texas MD Anderson Cancer and Mayo Clinic of Arizona.

Informed consent statement: As this is a retrospective review that involves no diagnostic or therapeutic intervention, as well as no direct patient contact, IRB permission was obtained with waiver of informed consent and waiver of authorization to use and

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Abstract

BACKGROUND

Primary pancreatic lymphoma (PPL) is a rare neoplasm. Being able to distinguish it from other pancreatic malignancies such as pancreatic ductal adenocarcinoma (PDAC) is important for appropriate management. Unlike PDAC, PPL is highly sensitive to chemotherapy and usually does not require surgery. Therefore, being able to identify PPL preoperatively will not only direct physicians towards the correct avenue of treatment, it will also avoid unnecessary surgical intervention.

AIM

To evaluate the typical and atypical multi-phasic computed tomography (CT) imaging features of PPL.

METHODS

A retrospective review was conducted of the clinical, radiological, and pathological records of all subjects with pathologically proven PPL who presented to our institutions between January 2000 and December 2020. Institutional review board approval was obtained for this investigation. The collected data were analyzed for subject demographics, clinical presentation, laboratory values, CT imaging features, and the treatment received. Presence of all CT imaging findings including size, site, morphology and imaging characteristics of PPL such as the presence or absence of nodal, vascular and ductal involvement in these subjects were recorded. Only those subjects who had a pre-treatment multiphasic CT of the abdomen were included in the study.

RESULTS

review patient information.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

Data sharing statement: No additional data is available.

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Twenty-nine cases of PPL were diagnosed between January 2000 and December 2020 (mean age 66 years; 13 males/16 females). All twenty-nine subjects were symptomatic but only 4 of the 29 subjects (14%) had B symptoms. Obstructive jaundice occurred in 24% of subjects. Elevated lactate dehydrogenase was seen in 81% of cases, whereas elevated cancer antigen 19-9 levels were present in only 10% of cases for which levels were recorded. The vast majority (90%) of tumors involved the pancreatic head and uncinate process. Mean tumor size was 7.8 cm (range, 4.0-13.8 cm). PPL presented homogenous hypoenhancement on CT in 72% of cases. Small volume peripancreatic lymphadenopathy was seen in 28% of subjects. Tumors demonstrated encasement of superior mesenteric vessels in 69% of cases but vascular stenosis or occlusion only manifested in 5 out of the twenty-nine individuals (17%). Mild pancreatic duct dilatation was also infrequent and seen in only 17% of cases, whereas common bile duct (CBD) dilation was seen in 41% of subjects. Necrosis occurred in 10% of cases. Size did not impact the prevalence of pancreatic and CBD dilation, necrosis, or mesenteric root infiltration (P = 0.525, P = 0.294, P = 0.543, and P = 0.097, respectively). Pancreatic atrophy was not present in any of the subjects.

CONCLUSION

PPL is an uncommon diagnosis best made preoperatively to avoid unnecessary surgery and ensure adequate treatment. In addition to the typical CT findings of PPL, such as homogeneous hypoenhancement, absence of vascular stenosis and occlusion despite encasement, and peripancreatic lymphadenopathy, this study highlighted many less typical findings, including small volume necrosis and pancreatic and bile duct dilation.

Key Words: Pancreatic tumors; Primary pancreatic lymphoma; Pancreatic ductal adenocarcinoma; Imaging features; Diagnosis; Computed tomography

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Core Tip: Primary pancreatic lymphoma (PPL) is often misdiagnosed as pancreatic adenocarcinoma. This two-center retrospective study of twenty-nine cases emphasized computed tomography (CT) imaging features useful for distinguishing PPL from its mimics. Distinct CT features of PPL, including a large homogenous hypovascular mass, absence of ductal dilation or atrophy, encasement of mesenteric vessels without invasion, and the presence of small volume peripancreatic adenopathy, should alert the clinicians to the potential diagnosis of this rare neoplasm. This study also demonstrated atypical findings such as necrosis and mild pancreatic or biliary ductal dilation should not rule out the diagnosis of PPL.

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INTRODUCTION

Primary pancreatic lymphoma (PPL) is an extremely rare malignancy, constituting less than 0.5% of pancreatic neoplasms and approximately 1% of all extranodal lymphomas[1-3]. The World Health Organization outlines PPL as a mass predominantly located in the pancreas with surrounding lymphatic node involvement[4]. In addition to these guidelines, Berhns' diagnostic criteria require (1) Normal white blood cell count; and (2) Nodal compromise defined within the peripancreatic region, without the presence of palpable superficial lymphadenopathy, mediastinal nodal enlargement, or hepatic and splenic involvement[5]. The clinical presentation of PPL often includes abdominal pain, weight loss, nausea and vomiting, and occasionally ob-

structive jaundice[4]. However, these nonspecific characteristics make it difficult to differentiate PPL from other pancreatic cancers, most notably the more common pancreatic ductal adenocarcinoma (PDAC)[2]. Furthermore, a paucity of information regarding PPL's distinctive radiologic findings makes diagnosis with imaging alone challenging[3].

Identifying unique preoperative characteristics of PPL so physicians may discriminate between PPL and PDAC early-on is crucial given their mutually exclusive prognoses and managements; while PDAC typically requires surgical intervention, the preferential treatment of PPL is chemotherapy with extensive follow-up due to this disease's high recurrence rate[3,4]. Therefore, this study aims to evaluate the common and uncommon computed tomography (CT) imaging appearances of PPL, as well as other diagnostic features that may enable definitive diagnosis.

MATERIALS AND METHODS

Study design

For this retrospective Health Insurance Portability and Accountability Act-compliant study, clinical, radiology, and pathology databases at the University of Texas MD Anderson Cancer Center, Houston, TX and Mayo Clinic, Phoenix, AZ were reviewed. This review searched the period between January 2000 and December 2020 for reports containing the strings "pancreas lymphoma" or "pancreatic lymphoma". Institutional review board permission was obtained for retrospective assessment of imaging, clinical, and pathologic data with waiver of informed consent from both the institutions involved in this study. The exclusion criteria were: (1) Lymphoma with mediastinal or pelvic adenopathy, bone marrow or hepatosplenic involvement which were considered to be systemic lymphoma and not PPL; (2) Pancreatic tumors suspected to be lymphoma on CT and later biopsy-proven to be another diagnosis on histological examination; (3) Subjects without a pretreatment multiphasic CT examination; and (4) Subjects whose medical records were not available of review.

The derivation of a cohort of 29 subjects is shown in Figure 1. All subjects had biopsy-proven PPL with a mass involving the pancreas. There were 13 males and 16 females with a mean age of 66 years (range, 55-88 years).

Imaging studies

Multislice CT scans of the abdomen were obtained in the pre-contrast, arterial, venous, and delayed phases. CT findings such as the size, site, morphology and imaging characteristics of PPL including the presence/absence of nodal, vascular and ductal involvement in these subjects were recorded by two reviewers (DG, KS) with 12 and 18 years of post-fellowship experience in Abdominal Imaging.

Statistical analysis

Descriptive statistics were compiled. Differences in sizes of tumors showing atypical findings (*i.e.,* pancreatic and bile duct dilation, mesenteric root infiltration, and tumor necrosis) and those without these findings was performed using the Mann-Whitney test. Frequency of these findings in tumors larger than 10 cm and less than 10 cm was performed with the Chi-Square test. MedCalc 19.3 (MedCalc Software Ltd, Ostend, Belgium) was used for statistical analysis.

RESULTS

Clinical presentation and management

The clinical presentation of the 29 biopsy-confirmed PPL cases used for imaging and clinical feature evaluation are summarized in Table 1. The most common presenting finding were vague abdominal pain (83%) and nausea and vomiting (31%). Weight loss was present in 21% of cases. Classic "B" symptom (*i.e.* fever, chills, night sweats, weight loss) were only seen in 14% of subjects. Similarly, 14% of cases demonstrated a palpable abdominal mass. Obstructive jaundice appeared in seven out of the twenty-nine subjects (24%).

All twenty-nine subjects underwent chemotherapy; the most common treatment was R-CHOP (given in 72% of cases). R-CVP was administered to 2 subjects (7%). In addition, 5 subjects (17%) underwent radiation therapy.

Table 1 Clinical presentation of primary pancreatic lymphoma

Characteristics	*n* (%)
Abdominal pain	24 (83)
Nausea and vomiting	9 (31)
Weight loss	6 (21)
Classic "B" symptoms	4 (14)
Palpable mass	4 (14)
Obstructive jaundice	7 (24)

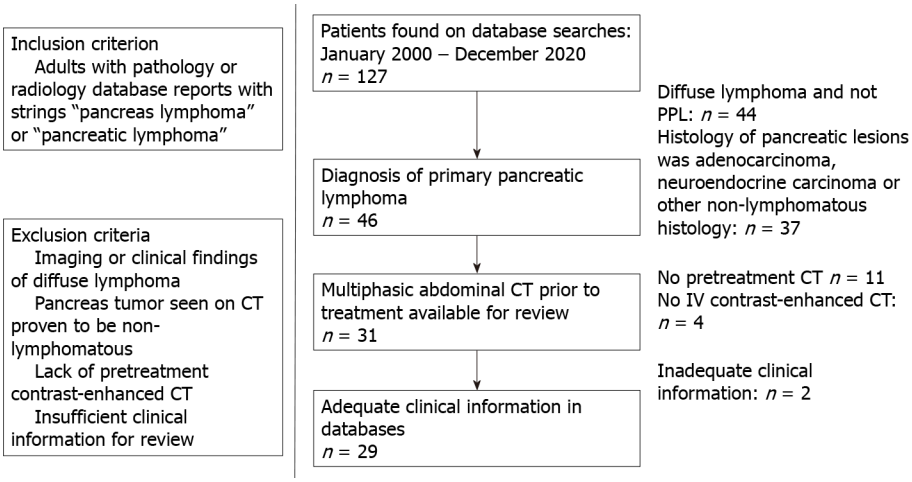


Figure 1 Inclusion and exclusion criteria of primary pancreatic lymphoma cohort. This figure gives the derivation of our cohort of 29 subjects for this study. CT: Computed tomography; PPL: Primary pancreatic lymphoma.

Laboratory findings

Lactate dehydrogenase (LDH) levels were elevated in 17 of the 21 cases for which LDH levels were recorded (81%). Elevated amylase was seen in four out of 11 subjects with recorded values (36%), all of whom presented with bile duct dilation (n = 3) or concomitant acute pancreatitis (n = 1). Cancer antigen 19-9 (CA 19-9) levels were within normal limits for 18 out of the 20 cases (90%). In the remaining two subjects biliary obstruction was present.

Imaging features

The prevalence of imaging findings are given in Table 2. The majority of tumors involved to the head and uncinate process (90%). Only four tumors (14%) occurred in the tail. Mean tumor size was 7.8 cm (range, 4.0-13.8 cm). Typical manifestation included a large (> 5 cm) infiltrative (52%) or focal, well-defined (48%) pancreatic mass, with homogenous hypoenhancement on post-contrast CT (72%) (Figure 2). In 41% of cases there was extension to the duodenum. Mass effect on the adjacent superior mesenteric vessels was seen in sixteen out of the twenty-nine subjects (55%). In addition, the majority of cases (69%) demonstrated encasement of superior mesenteric artery (SMA), superior mesenteric vein (SMV), or both; however, only five subjects (17%) had vascular stenosis or occlusion (Figure 3). In one subject there was active bleeding into the tumor by erosion of a mesenteric artery branch leading to intratumoral hematoma (Figure 4).

Furthermore, pancreatic ductal dilation was absent in 83% of cases; the remaining five subjects demonstrated mild dilation with duct caliber of less than 5 mm (Figures 3 and 5). There was no difference in size of those without and with pancreatic duct dilation (median sizes of 6.9 *vs* 6.8 cm, P = 0.525) or in the frequency of pancreatic duct dilation between tumors greater than 10 cm and less than 10 cm (P = 0.366). Common bile duct (CBD) dilation was seen in 41% of cases (Figure 3). Tumor size did not differ between those without and with CBD dilation (median sizes of 5.4 *vs* 7.1 cm, P = 0.294). There was no difference in frequency of CBD dilation between tumors greater

Table 2 Imaging features of primary pancreatic lymphoma

Characteristics	*n* (%)
Location	
Head	23 (80)
Body	3 (10)
Tail	4 (14)
Uncinate process	16 (55)
Mean size (cm)	7.8
Infiltrative mass	15 (52)
Focal mass	14 (48)
Homogeneous hypoenhancement	21 (72)
Tumor necrosis	3 (10)
Extension to nuodenum	12 (41)
Mass effect on SMV	16 (55)
Encasement of SMA/SMV	20 (69)
Vascular stenosis/occlusion	5 (17)
Pancreatic ductal dilation	5 (17)
Common bile duct dilation	12 (41)
Pancreatic atrophy	0 (0)
Infiltration of mesenteric root	4 (14)
Peripancreatic lymphadenopathy	8 (28)

SMV: Superior mesenteric vein; SMA: Superior mesenteric artery.

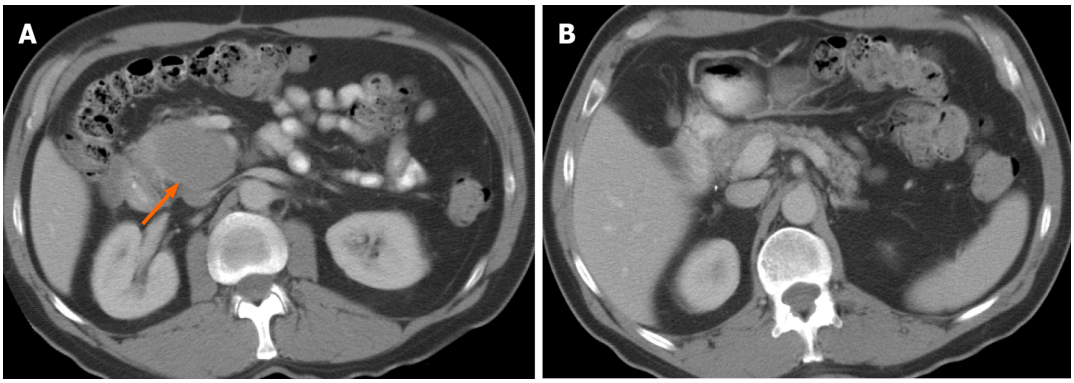


Figure 2 A 58-year-old male with abdominal pain. A: Axial computed tomography images demonstrate a large, relatively well-defined focal mass (arrow) involving the uncinate process of the pancreas; B: Axial computed tomography images. The tumor shows homogenous hypoenhancement, without evidence of any necrosis or calcification. The rest of the pancreas is preserved, and there is no pancreatic ductal dilation. Biopsy confirmed diagnosis of primary pancreatic lymphoma.

than 10 cm and less than 10 cm (P = 0.823)

No cases presented signs of pancreatic atrophy. Peripancreatic lymphadenopathy manifested in eight subjects (28%), five of which included involvement below the renal vein. These nodes all had a short-axis dimension of less than 1.5 cm. Mesenteric root infiltration was seen in 14% of cases (Figures 3 and 5). There was no significant difference in tumor size of those without and with mesenteric root infiltration (median sizes of 6.8 *vs* 12.8 cm, P = 0.097), although there was an overall trend for larger tumors to infiltrate the mesentery.

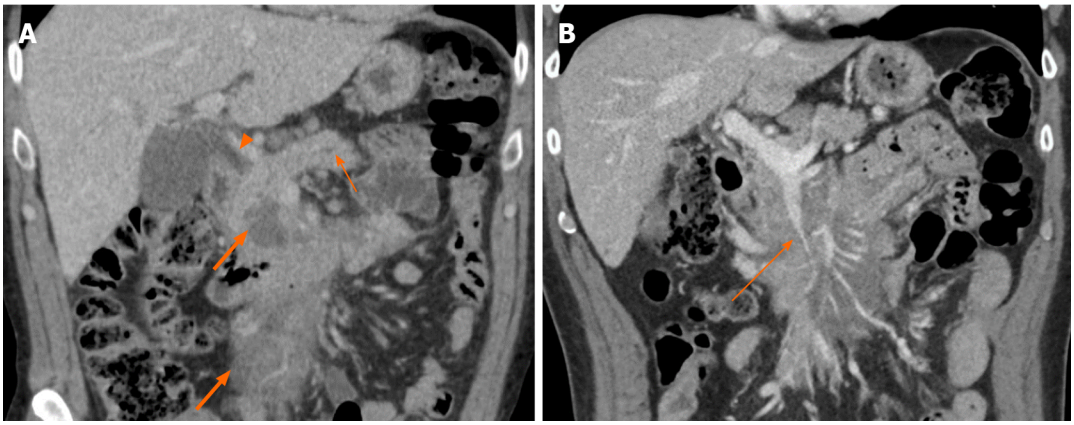


Figure 3 A 72-year-old male presenting with abdominal pain and jaundice. Coronal computed tomography imaging shows hypoenhancing homogenous infiltrative mass (thick arrows, A) infiltrating the mesenteric root. A: There is pancreatic duct dilation (short thin arrow) and common bile duct obstruction (arrowhead); B: There is stenosis of the superior mesenteric vein (long thin arrow) caused by the tumor.

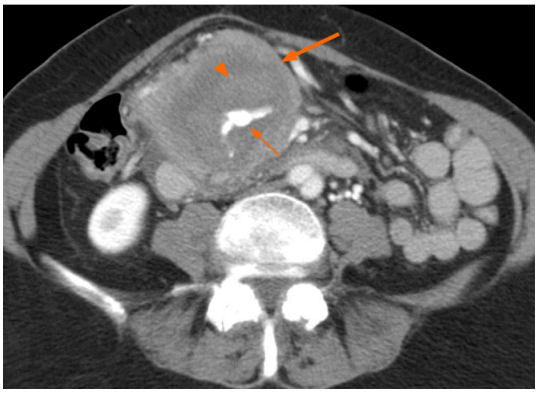


Figure 4 A 74-year-old female presenting with abdominal pain and nausea. Axial computed tomography shows mass (thick arrow) encircling an aneurysmal vessel (thin arrow) which is bleeding into the mass, causing hematoma (arrowhead). The tumor was biopsy-proven to be primary pancreatic lymphoma.

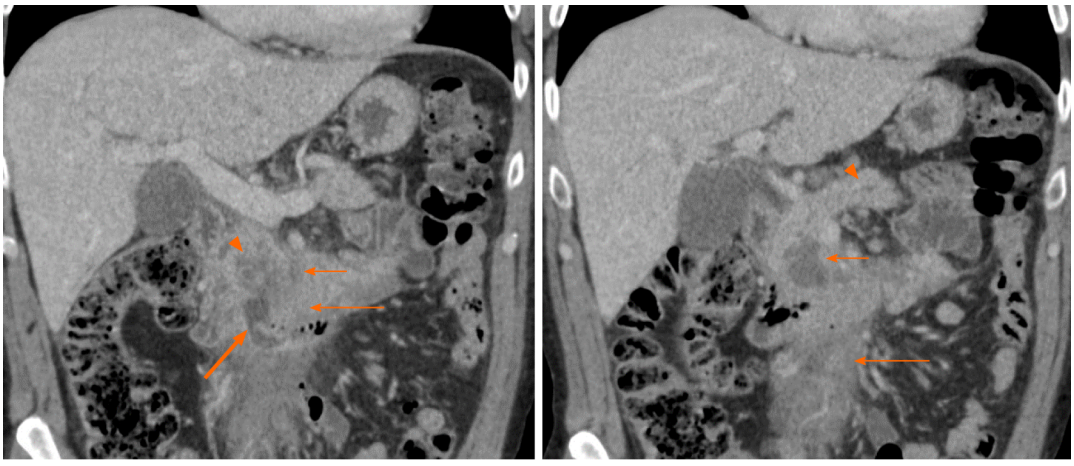


Figure 5 A 62-year-old male presenting with abdominal pain. Coronal computed tomography images show low-density necrosis (thin short arrow) within tumor (thin long arrow), which infiltrates the mesenteric root without occlusion of mesenteric vessels. There is a collection extending from the third part of the duodenum into tumor (thick arrow) suggesting fistula from probable erosion of duodenal wall. Mild pancreatic duct dilation is seen (arrowhead).

Small foci of low density within tumor less than 10% of volume, and likely to be necrosis, was seen in three cases (10%). These subjects either had acute pancreatitis (n = 2) or a duodenal fistula (n = 1) (Figure 5). There was no significant difference in size of those without and with necrosis (median sizes of 7.0 *vs* 6.2 cm, P = 0.543) or in frequency of necrosis between tumors greater than 10 cm and less than 10 cm (P =

0.950).

DISCUSSION

In agreement with prior literature, subjects in this study presented with nonspecific abdominal symptoms including vague epigastric pain, nausea and vomiting, weight loss, obstructive jaundice, and a palpable mass, making PPL difficult to distinguish from other pancreatic pathologies based on clinical presentation alone[4,6]. Out of these presentations, the most prevalent in PPL are abdominal pain, nausea and vomiting, and weight loss. The decreased prevalence of obstructive jaundice among PPL cases in comparison to individuals with PDAC could aid differentiating between the two; nevertheless, obstructive jaundice is still a relatively common potential presentation of PPL[7-10]. Classic "B" symptoms such as fever, chills, night sweats, and weight loss associated with systemic non-Hodgkin lymphoma are rare in PPL, and only occurred in 4 cases from this study[4].

Laboratory values may play a more decisive role in the diagnosis of the PPL. Elevated tumor marker CA 19-9 has been reported in 80% of PDAC cases but is a rare finding of PPL[4]. In this study, CA 19-9 was within normal limits for the vast majority of subjects. PPL cases with biliary dilation may be associated with mild elevation of CA 19-9[11]. In keeping with this study, elevated amylase has been observed in PPL by other studies and numerous case reports, often due to associated acute pancreatitis or biliary obstruction[8-10,12,13]. Other useful biomarkers include LDH which is often elevated in non-Hodgkin lymphomas, including PPL, but is less likely to be elevated in other pancreatic neoplasms such as PDAC[10,11].

Despite the potential use of laboratory values to suggest PPL over its mimics, the overall nonspecificity of clinical and laboratory features of PPL emphasizes the importance of imaging. CT is the most common imaging study performed for diagnosis and characterization of pancreatic masses[9]. PPL typically manifests as a diffusely infiltrative or focal, well-defined mass with homogenous hypoenhancement on post-contrast CT (Figure 2)[14,15]. In particular, the mass-like appearance can often be mistaken for PDAC. However, one CT feature that suggests PPL over PDAC is encasement of the SMA and/or SMV without narrowing or occlusion; conversely, locally advanced PDAC invades or stenoses these vessels[4,6]. In this study, 69% of cases demonstrated encasement of the SMA, SMV or both, but significantly less had vascular invasion or occlusion.

Lymphadenopathy localized to the peripancreatic region also suggests PPL[16]. In particular, the presence of lymphadenopathy below the level of renal hilum may be useful in excluding the diagnosis of PDAC[9,17,18]. The majority of cases in this study demonstrating lymphadenopathy presented nodal involvement below the renal hilum. In addition, all nodal involvement in this study was less than 1.5 cm in short-axis diameter, which may be useful for differentiating PPL from diffuse lymphoma secondarily involving the pancreas[19].

Perhaps the most important findings which indicate PPL rather than PDAC are the absence of pancreatic ductal dilation and pancreatic atrophy; only 17% of subjects in this study showed mild pancreatic ductal dilation (< 5 mm) and no subjects presented pancreatic atrophy (Figures 3 and 5)[14]. This is a useful differentiating feature as even small pancreatic adenocarcinoma located in the head of pancreas tends to result in moderate or severe upstream pancreatic ductal dilatation as well as pancreatic atrophy [4,6,20]. Nevertheless, mild pancreatic duct dilation may still be seen in PPL and should not be used to exclude this diagnosis.

In addition to cases of occasional mild pancreatic ductal dilation, this study has demonstrated multiple atypical features of PPL that have not been widely reported. Interestingly, CBD dilation was seen in a substantial proportion (41%) of subjects. While not widely reported in studies on PPL, Boninsegna *et al*[21] found similar results to this study, finding CBD dilation in six out of the fourteen subjects (43%) included in their series.

Ten percent of cases in this cohort showed partial necrosis of the tumor. This finding is contrary to the existing consensus that the presence of necrosis effectively excludes a diagnosis of PPL[5,7,11,17]. While most PPL do not present necrosis, this study demonstrates that complications such as concomitant acute pancreatitis or intratumoral fluid collection from a duodenal fistula may lead to this atypical finding (Figure 5).

To our knowledge, infiltration of the mesenteric root by PPL has not been reported by previous studies. This finding was demonstrated in 14% of cases in this study

(Figures 3 and 5). As expected, the mesenteric vessels were not thrombosed or occluded; however, in one case stenosis of the SMV was seen (Figure 3). There was active extravasation of blood into the tumor in one subject, likely as a result of tumor erosion of the vessels wall (Figure 4).

There are limitations of this study, including its retrospective design. In addition, PPL is a rare entity, and the combined databases of two major institutions only revealed 29 cases of biopsy-proven PPL with pretreatment multiphase CT. Nevertheless, this study revealed useful data on the differentiating features of PPL, and less typical findings which may occur.

CONCLUSION

PPL is a rare tumor, which may be misdiagnosed as PDAC, but differentiation between the two entities is critical in order to avoid unnecessary surgery and associated morbidity and mortality. CT imaging features, combined with clinical presentation and laboratory work, can help improve the diagnosis of PPL. The presence of a large relatively homogenous hypoenhancing mass involving the pancreatic head, absence of pancreatic ductal dilatation or atrophy, lack of vascular stenosis or occlusion despite encasement, and presence of small volume peripancreatic lymphadenopathy are helpful features for diagnosing PPL. However, unusual features such as tumor necrosis and the presence of mild pancreatic or bile duct dilation should not automatically exclude PPL as a potential diagnosis. It is important that clinicians are aware of both typical and atypical findings in order to raise the possibility of PPL without delay in diagnosis.

ARTICLE HIGHLIGHTS

Research background

Primary pancreatic lymphoma (PPL) is a rare neoplasm. The ability to differentiate PPL from other pancreatic malignancies including pancreatic ductal adenocarcinoma (PDAC) is important for appropriate management. However, the nonspecific characteristics currently associated with PPL and a lack of information regarding PPL's distinctive imaging features makes diagnosis difficult.

Research motivation

Identifying typical and atypical features of PPL on computed tomography (CT), as well as other diagnostic features that may differentiate PPL from its mimics, may enable definitive diagnosis. The discovery of features which distinguish PPL from PDAC early-on is critical to avoid unnecessary surgery.

Research objectives

This study aims to evaluate the typical and atypical CT imaging appearances of PPL. In addition, it distinguishes various clinical and laboratory markers which may be useful to identify PPL. An emphasis was placed on differentiating PPL from PDAC, which can be difficult to do using the current characteristics associated with PPL.

Research methods

Radiology, clinical, and pathology databases from two institutions were searched for reports between January 2000 and December 2020 containing the strings "pancreas lymphoma" or "pancreatic lymphoma". The exclusion criteria were: (1) Lymphoma with mediastinal or pelvic adenopathy, bone marrow or hepatosplenic involvement which were considered to be systemic lymphoma and not PPL; (2) Pancreatic tumors suspected to be lymphoma on CT and later biopsy-proven to be another diagnosis on histological examination; (3) Subjects without a pretreatment multiphasic CT examination; and (4) Subjects whose medical records were not available of review. Multislice CT scans of the abdomen were viewed in the pre-contrast, arterial, venous, and delayed phases. The clinical presentation, management, laboratory findings, and imaging features of all included patients (n = 29) were evaluated.

Research results

All twenty-nine subjects were symptomatic, but only 14% demonstrated B symptoms

and 24% demonstrated obstructive jaundice. Lactate dehydrogenase (LDH) levels were elevated in 17 of the 21 cases for which LDH levels were recorded (81%), however cancer antigen 19-9 (CA 19-9) levels were within normal limits for 18 out of the 20 cases for which values were recorded (90%). Pancreatic ductal dilation was absent in 83% of cases and no patients presented pancreatic atrophy. Atypical features of PPL included pancreatic bile duct dilation (17%), common bile duct (CBD) dilation (41%), necrosis (10%), and infiltration of the mesenteric root (14%). Size did not impact the prevalence of pancreatic and CBD dilation, necrosis, or mesenteric root infiltration (P = 0.525, P = 0.294, P = 0.543, and P = 0.097, respectively).

Research conclusions

The decreased prevalence of obstructive jaundice, elevated CA 19-9 levels, pancreatic ductal dilation, and pancreatic atrophy, as well as the increased elevation of LDH levels, encasement of the small mesenteric artery and/or vein without invasion or stenosis, and lymphadenopathy limited to the peripancreatic region, may be useful for distinguish PPL from its mimics, such as PDAC. However, in addition to the occasional appearance of pancreatic ductal dilation, this study identified multiple atypical features of PPL including the presence of CBD dilation, necrosis, and infiltration of the mesenteric root.

Research perspectives

Prospective studies with larger cohorts must be conducted to support the findings of this paper and the potential use of its highlighted imaging and clinical features for definitive diagnosis of PPL. In addition, there is a need for direct comparison of the frequency of these features in PPL *vs* PDAC, to determine how useful they are in differentiating the two entities.

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EVIDENCE REVIEW

Postoperative radiotherapy in resected non-small cell lung cancer: The never-ending story

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Abstract

This manuscript collects in a joint and orderly manner the existing evidence at the present time about postoperative treatment with radiotherapy in non-small cell lung cancer. It also systematically reviews the current evidence, the international recommendations in the most relevant guidelines, the most controversial aspects in clinical and pathological staging, the specific technical aspects of radiotherapy treatment, and also collects all the potential risk factors that have been postulated as significant in the prognosis of these patients, evaluating the possibility of segmenting a particularly sensitive subpopulation with a high risk of relapse on which an adjuvant treatment with radiotherapy could have an impact on their

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clinical evolution. Finally, currently active trials that aspire to provide more evidence on this topic are reviewed.

Key Words: Non-small lung cancer; Radiotherapy; Postoperative; Lung cancer

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Core Tip: The approach to the postoperative treatment of non-small cell lung cancer (NSCLC) is one of the pending subjects of the specialty of Radiation Oncology. Despite the enormous anticipation that the Lung-Art trial had produced, its results leave issues unresolved. In this article, we attempt to systematically recapitulate the currently existing evidence for the radiotherapeutic management of this pathology, in order to identify those patients who could potentially benefit more from postoperative treatment in NSCLC.

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INTRODUCTION

Historical evolution of postoperative radiotherapy

One of the great historical controversies in the field of thoracic oncology is the use of Postoperative radiotherapy (PORT) in patients with non-small cell lung cancer (NSCLC). The rationale for this therapeutic strategy is the high risk of locoregional recurrence (LRR) after radical surgery, especially in patients with pN2 disease, who account for up to 30% of patients. The development of LRR in patients with NSCLC has important clinical implications and is associated with worse survival outcomes[1]. Several different pathological variables have been associated with a higher risk of developing LRR, including tumour size > 3 cm, lymphovascular invasion, visceral pleural invasion, and involvement of multiple lymph nodes[2].

The role of PORT in NSCLC remains controversial, mainly because studies carried out over the last few decades have reported conflicting safety and efficacy results. Although multiple retrospective and prospective studies have been performed, we still lack high-quality evidence to confirm or definitively rule out PORT in these patients. A meta-analysis published in 1998 found that PORT was associated with lower overall survival (OS) rates in patients with stage I-II disease, with 2-year OS rates of 43% in the non-PORT group *vs* 30% in the patients that received PORT, although there was no clear evidence that PORT negatively influenced outcomes in patients with stage III pN2 disease[3]. In older studies, the poor outcomes of PORT could be due to the high levels of morbidity and mortality associated with obsolete radiotherapy techniques or inappropriate doses, fractionations, and/or irradiation volumes. In fact, a more recent meta-analysis demonstrated that PORT improves OS outcomes when modern technology (linear accelerators *vs* cobalt therapy units) is used to deliver the radiation dose[4].

Despite the contradictory findings described above, several studies have reported a clear benefit for PORT in patients with involved lymph nodes (pN2) in terms of improved local control and even OS[5-7]. Among those studies with positive findings, the most important is the study carried out by Mikell *et al*[7], who evaluated 2115 patients with pN2 NSCLC based on data retrieved from the National Cancer Database (NCBD). In that study, PORT was associated with a significant increase in OS (42 mo *vs* 38 mo, $P = 0.048$) in patients treated according to the therapeutic standards of the modern era [three-dimensional conformal radiotherapy (3D-CRT), adjuvant chemotherapy (ChT), *etc.*][7].

The long-awaited preliminary results of the Lung ART trial (NCT00410683)[8], which included patients with NSCLC who underwent complete resection with adjuvant ChT, were recently presented at the ESMO 2020 meeting. Lung ART is a

multi-institutional randomized phase III trial which included stage III N2 NSCLC cases comparing mediastinal PORT (54 Gy/27-30 fractions) to no PORT in very selected patients: PS 0-2, complete resection with optimal nodal exploration and proven N2 disease. The main endpoint was disease-free survival (DFS). Between August 2007 and July 2018, 501 patients were randomized after surgery or after ChT: 252 patients allocated to PORT, and 249 to no PORT. With a median FU of 4.8 years DFS HR was 0.85 (95%CI [0.67-1.07]); median DFS was 30.5 mo with PORT and 22.8 without PORT; 3-year DFS was 47.1% with PORT *vs* 43.8% without PORT (*P* = ns), and finally, 3-year OS was 66.5% with PORT *vs* 68.5% without PORT (*P* = ns). Early and late Gr 3-5 cardio-pulmonary toxicity was respectively 7% and 20% in PORT arm *vs* 3.2% and 7.7% in control arm. Nonetheless, PORT significantly decreased LRR in the mediastinum (46.1% *vs* 25% with and without PORT, respectively), a finding that suggests that PORT could offer a clinical benefit in a well-selected subgroup of patients.

However, these preliminary results raised further doubts about the role of PORT in NSCLC. The findings of this landmark trial are extremely important and may come to redefine the role of radiotherapy in NSCLC.

According to these data, PORT should not be routinely recommended to all resected stage III N2 NSCLC patients. The decision to prescribe o not PORT must be individualised according to the patient's specific characteristics. In general, PORT should be indicated only in highly selected patients with good performance status (PS 0-1), significant mediastinal lymph node involvement (pN2, extracapsular extension), and/or residual disease (R1-R2) after surgery. In addition, PORT must be only performed in cases with a favourable dose distribution that fulfils the dose restriction criteria for the organs of risk (OARs), especially cardiopulmonary restrictions.

CURRENT EVIDENCE AND RECOMMENDATIONS FOR PORT

The role of PORT in the treatment of NSCLC remains controversial. Although this therapeutic strategy has been evaluated in numerous retrospective and prospective studies, robust evidence to definitively support the value of PORT is still lacking, as can be seen in the lack of consensus among the clinical guidelines published by the main international scientific societies[9-13].

Currently, the most widely accepted indication for PORT, with the most evidence, is for the treatment of residual disease (including extracapsular extension) after radical surgery. Most international guidelines recommend PORT in patients with involved surgical margins (R1-R2) at the surgical bed due to the high risk of recurrence in this region, with a recommended dose ranging from 54-60 Gy (1.8-2 Gy/fraction)[14].

By contrast, in patients with stage pN2 disease, the current evidence suggests that the treatment decision should be assessed on a case-by-case basis by a multidisciplinary team to determine if the patient would be likely to benefit from PORT. The treatment decision should consider several key clinical characteristics, including the number of mediastinal nodal stations involved (≥ 1), the patient's general physical condition (PS 0-1), and cardiopulmonary function. Table 1 summarizes the recommendations proposed by the main international guidelines.

MANAGEMENT OF CASES WITH INVOLVED SURGICAL MARGINS

The rate of incomplete resections (microscopic or macroscopic; R1-R2) after radical surgery for lung cancer ranges from 1%-17%[15]. In these cases, the aim of PORT is to reduce the risk of local recurrence and improve OS. Although various clinical guidelines recommend salvage surgery in patients with positive surgical margins, this approach is not supported by robust data. Ghiribelli *et al*[16] evaluated OS in a series of patients with incomplete resections (R1), finding that survival was not correlated with the type of infiltration, nodal involvement, or histological type. As a result, in patients with microscopic residual tumours, the authors recommended salvage surgery only in patients with early stage (I-II) disease; by contrast, the recommended treatment in stage III pN2 disease is adjuvant radiotherapy.

A study published in 2012 evaluated the efficacy and toxicity of PORT according to histological subtype in patients (*n* = 41) with incompletely resected NSCLC[17]. Of the 41 patients, 23 had microscopic (R1) and 18 macroscopic (R2) residual disease. The histologic distribution was as follows: squamous cell carcinoma (SCC) (*n* = 23), adenocarcinoma (14), and other histologies (4). The predominant progression pattern

Table 1 Recommendations for postoperative radiotherapy according to the main international guidelines

Guidelines	Clinical scenario	Recommendation for PORT
NCCN[9]	Stage pN0-1	Not recommended
	Stage pN2, negative surgical margins (R0)	Sequential
	Microscopic or macroscopic surgical margins (R1-R2)	Concomitant (selected cases) or sequential
ASTRO[10]	Stage pN2	Sequential
	Microscopic or macroscopic surgical margins (R1-R2)	Concomitant (selected cases) or sequential
	ESTRO-ASTRO[11]	
	Multiple nodal stations involved	Sequential
	Extracapsular nodal extension	Sequential
ESMO[12]	Early stage (I-II) disease (R0)	Not recommended
	Positive margins or chest wall involvement (R1-R2)	Sequential
	Stage pN2	Only in selected cases
ASCO[13]	Early stage (I-II) disease (R0)	Not recommended
	Stage pN2	Only in selected cases

NCCN: National Comprehensive Cancer Network; ASTRO: American Sociedad of Radiation Oncology; ESTRO: European Society for Radiotherapy & Oncology; ESMO: European Society for Medical Oncology; ASCO: American Society of Clinical Oncology; PORT: Postoperative radiotherapy.

was distant disease, observed in 13% of patients with SCC and 64% of those with adenocarcinoma ($P < 0.01$). Survival rates at 5-years were as follows: OS, 56%; local control (LC), 63%; DFS, 37%; and metastasis-free survival (MFS), 49%. On the multivariate analysis, the only significant predictors of better survival (DFS and MFS) were SCC histology, stage N0-1, and R1 surgical margins. The authors concluded that, in patients with R1 margins, PORT provides good LC without severe toxicity, but systemic therapy should always be considered due to the high risk of distant metastasis.

Hancock *et al*[18] evaluated 3102 surgically treated NSCLC patients included in the NCDB registry. Of these, 1688 had microscopically positive margins (R1). The authors compared patients according to margin status (R1 *vs* R0), with significantly lower 5-year OS rates in the R1 group for all stages: stage I, 37% *vs* 62% (P <0.0001); stage II, 29% *vs* 41% ($P < 0.0001$); and stage III, 19% *vs* 33% ($P < 0.0001$). Administration of adjuvant ChT with PORT in the R1 group was associated with better OS than surgery alone, regardless of stage (stage I, 44% *vs* 35%, $P = 0.05$; stage II, 33% *vs* 21%, $P = 0.0013$; stage III, 30% *vs* 12%, $P < 0.0001$).

In a study published in 2015, Wang *et al*[19] evaluated 3395 patients with incompletely resected stage II-III NSCLC to determine the influence of PORT on survival outcomes, finding that PORT was associated with significantly better 5-year OS (32.4% *vs* 23.7%). Radiation doses between 50-70 Gy improved survival rates in the PORT group *vs* the non-PORT group. However, when higher doses (> 70 Gy) were administered, there were no between-group differences in OS. The authors of that study concluded that PORT improves OS in patients with incompletely resected stage II-III NSCLC and should therefore be considered as an adjuvant treatment. They also suggested that the radiation dose in patients with macroscopic residual disease (R2) should be the same as those used for radical radiotherapy (60-66 Gy).

MEDIASTINAL STAGING

Preoperative mediastinal staging

The appropriate management of NSCLC depends on accurate mediastinal staging. Contrast-enhanced chest computed tomography (CT) is currently the diagnostic test of choice for preoperative mediastinal staging. On CT imaging, nodes with a short-axis diameter ≥ 1 cm are considered pathological[20]. In recent years, 18F-fluorodeoxy-glucose (FDG) positron-emission tomography (PET)-CT has transformed lung cancer staging due to its greater sensitivity. However, PET-CT has some limitations in cases

with small nodes (< 1 cm) and in certain histologies in which FDG uptake is limited. PET-CT also has a high false positive rate (20%-25%) in the presence of intercurrent infections and inflammatory processes. Consequently, histopathologic confirmation of mediastinal node involvement is usually required, especially when the therapeutic approach depends directly on the results of this assessment[21-23]. Histological confirmation can be omitted in certain patients with small (≤ 3 cm) peripheral tumours without radiological evidence of suspected mediastinal involvement.

Mediastinal nodes can be obtained endoscopically through endobronchial ultrasound (EBUS) and/or endoscopic ultrasound (EUS) guided puncture, or surgically, through mediastinoscopy or video-assisted thoracoscopy (VATS). Endobronchial ultrasound (EBUS/EUS) is usually the first step in evaluating suspected mediastinal node involvement[24,25]. These minimally invasive endoscopic techniques are usually preferred to surgical approaches due to their good sensitivity and specificity profile and relatively low risk of morbidity. If the sample is negative, not assessable, or insufficient (despite radiological suspicion), staging should be completed with invasive techniques, which have a higher negative predictive value (NPV). For many years, conventional mediastinoscopy was the main surgical staging technique, despite the technical limitations of this procedure for the study of the posterior and inferior mediastinum, in which either extended cervical mediastinoscopy or VATS is necessary[26].

Mediastinal restaging after neoadjuvant therapy

Mediastinal restaging after neoadjuvant therapy (ChT or ChT+RT) is controversial. Some patients with stage IIIA, low volume N2 disease are classified as potentially resectable and may benefit from neoadjuvant therapy, which could increase the likelihood of achieving a complete response (CR) in the mediastinum, thus permitting surgical resection of the tumour[27]. In this clinical scenario, however, the value of CT for mediastinal restaging is questionable since CT-based assessment, although highly predictive of pathologic CR, tends to underestimate the true CR rate.

PET-CT is an excellent tool to assess the response of both the primary tumour and metastatic lesions, but it is less reliable in evaluating mediastinal involvement due to high rates of false negative and false positives (20% and 25%, respectively)[28,29]. Therefore, histopathologic confirmation is necessary in cases with radiological response if surgical resection is being considered.

EBUS/EUS restaging after neoadjuvant therapy has a low sensitivity and a low NPV. If the test is negative, the surgical technique should be escalated to reduce the false negative rate[30]. Restaging *via* mediastinoscopy has a high sensitivity (> 60%), specificity (≈ 100%), positive predictive value (PPV; 100%) and NPV (> 73%); however, this procedure is not routinely performed due to its technical complexity in this clinical context. Rather, the recommended strategy is initial confirmation of stage N2 disease by EBUS or EUS-guided transbronchial aspiration during the initial workup, thus reserving mediastinoscopy for restaging[31].

SELECTION OF CANDIDATES FOR PORT

Numerous studies have explored a wide range of prognostic factors potentially associated with an increased risk of LRR in order to identify high-risk patients suitable for adjuvant radiotherapy. In patients with NSCLC, the histological type is not currently considered a prognostic factor for adjuvant treatment due to the poor quality of the available data and contradictory findings in the literature. While some studies have found that SCC histology is associated with worse OS rates than adenocarcinoma [32,33], findings from other studies point in the opposite direction[34].

The findings of a recent meta-analysis involving 25780 patients from 13 studies (most retrospective) underscored the prognostic value of multiple mediastinal node involvement. That study showed that, in patients with pN2 disease with ≥ one positive node and/or multiple N2 station involvement, PORT significantly improved both DFS (HR 0.57, 95% confidence interval [CI], 0.38–0.85) and OS (HR 0.85, 95%CI, 0.79–0.92) [35].

The lymph node ratio (LNR)—defined as the number of involved nodes divided by the total removed or examined—has also been significantly associated with survival outcomes. A recent study evaluated 11341 patients with NSCLC and postoperative nodal involvement included the SEER (Surveillance, Epidemiology, and End Results Database) registry. The authors established three risk categories according to the LNR (LNR1 ≤ 0.28, LNR2 < 0.81, and LNR3 > 0.81), finding that LNR3 was an independent

prognostic factor for cancer-specific survival (CSS) (HR 2.54; 95%CI, 2.30–2.80; $P < 0.001$)[36].

Other parameters, such as the positive and negative lymph node counts (PLN and NLN, respectively), have been developed to quantify the tumour load in mediastinal nodes. Zhou *et al*[37] reviewed data from 39959 surgically-treated cases of NSCLC, demonstrating a significant association between mediastinal tumour burden and OS (PLN > 5; HR 2.0128, 95%CI: 1.6996–2.3836; NLN > 5; HR 0.7493, 95%CI: 0.7211–0.7785; LNR > 0.30; HR 1.7949, 95%CI: 1.5329–2.1016); and with CSS (PLN > 5; HR 2.2147, 95%CI: 1.8095–2.7106; NLN > 5; HR 0.7214, 95%CI: 0.6869–0.7575; LNR > 0.30; HR 1.9627, 95%CI: 1.6219–2.3752). In this same line of research, another study evaluated 5168 patients with stage IIIA-N2 NSCLC, finding that patients with PLN > 5 who underwent PORT had significantly better OS outcomes (HR 0.637, 95%CI: 0.518–0.784), a benefit that persisted even when compared to adjuvant ChT alone (HR 0.726, 95%CI: 0.564–0.934)[38].

The studies that have generated the most interest are those that have sought to stratify risk groups according to multiple clinical, pathologic, and molecular parameters. In this regard, the study by Deng and colleagues[39] is worth highlighting. Those authors evaluated numerous characteristics — age, sex, surgical technique, histological type, degree of differentiation, tumour size, number of nodes evaluated (LNR index) — in a large sample ($n = 2329$) of patients included in the SEER database. Based on that analysis, the authors proposed a prognostic scoring model that classified patients into two risk categories (high and low), which was a significant predictor of survival outcomes (OS and CSS)[40].

Jiang *et al*[40] recently developed a model that incorporated several molecular biomarkers, together with other well-known clinical variables, to predict clinical outcomes in patients with stage IIIA pN2 NSCLC. In that study, the following variables were significantly associated with the risk of LRR: epidermal growth factor receptor (EGFR) status: wild-type *vs* native (HR 3.666, 95%CI: 1.724-7.797); lymphocyte to monocyte ratio (LMR) < 4.69 (HR 2.364, 95%CI: 1.221-4.574); surgical procedure (VATS *vs* thoracotomy) (HR 0.348, 95%CI: 0.175 -0.693); and pN2 LNR ≥ 38.9% (HR 3.597, 95%CI: 1.832-7.062). The authors then used those data to develop a predictive model (Table 2) based on the four independent risk factors to determine the individual risk of LRR in each patient. This score, in turn, could be used to recommend or not adjuvant radiotherapy[41].

TECHNICAL RECOMMENDATIONS FOR THE TREATMENT OF PORT

Simulation

The generally accepted recommendations provided by clinical guidelines for the management of NSCLC should be followed for positioning, immobilization, and treatment simulation. Systems designed to improve immobilization and control respiratory motion (4D-CT) should be used, preferably with image-guided radiotherapy (IGRT), to obtain smaller treatment volumes and more precise radiotherapy to achieve a better dosimetric distribution.

In general, CT imaging (slice thickness, 2-3 mm) should be performed with intravenous contrast to improve contouring of the nodal areas[42,43]. The use of 5FDG-PET-CT for postoperative simulation is not recommended due to the lack of robust data; moreover, interpretation of these images in the immediate postoperative period can be challenging due to the inflammation, which can lead to false positives. Image interpretation after ChT is also difficult and it is easy to underestimate the residual disease (false negatives)[44].

Target volumes

The most important data for target volume definition were described in the Lung-ART clinical trial and based on contouring performed by 17 experienced thoracic radiation oncologists in two representative cases[45]. The clinical target volume (CTV) should include the bronchial stump, ipsilateral hilum, adjacent mediastinal pleura, and involved nodes (according to the pathology report). The involved nodal station and those immediately superior and inferior to that region should also be contoured, being careful to avoid oversizing the CTV. To generate the PTV (planning target volume), a margin of at least 0.5 cm in the mediolateral and dorsoventral directions (1 cm in the craniocaudal direction) should be applied to the CTV to minimize uncertainties related to tumour motion and patient positioning[46].

Table 2 Proposed predictive model for locoregional recurrence in stage IIIA N2 non-small cell lung cancer[41]

Risk model for LRR in stage pIIIA-N2 NSCLC		
Factor	**Category**	**Score**
EGFR status	Wild- type	4
LMR	LMR < 4.69	2
Type of surgery	Thoracotomy	3
LNR	LNR ≥ 38.9	4
Risk group	**Score**	**3-yr LRFS**
Low risk	0-2	71.4%
Medium risk	3-5	57.3%
High risk	6-13	13.6%

LRFS: Locoregional recurrence-free survival; LRR: Locoregional recurrence; NSCLC: Non-small cell lung cancer; LNR: Lymph node ratio; LMR: Lymphocyte-to-monocyte ratio.

The definition of critical organs (OARs)[47] and dose restrictions are the same as in NSCLC, although with more restrictive lung criteria. In post-lobectomy patients, Boonyawan *et al*[47], proposed limiting the lung volume that receives 10 and 20 Gy (V10 and V20) to < 30% and < 20%, respectively[48]. In patients older than age 65, the lung V5 should be reduced to ≤ 36%[49]; if IMRT is performed, the recommended V5 is < 64.9%, with mean lung dose (MLD) < 10.8 Gy[50]. In patients undergoing pneumonectomy, to ensure safety, these limitations should be even more restrictive, as follows: V5 < 30%, V20 < 13%, and MLD < 7.5 Gy[51]. If 3D-CRT is used, the V20 should be < 10%[52].

Dose and fractionation

In completed-resected (R0) surgeries, the recommended dose is 50-54 Gy using a conventional fractionation scheme (1.8-2 Gy/d)[53]. However, in high risk patients with R1 or R2 margins, the total dose may be increased up to 54-60 Gy, or even up to radical doses of 60-66 Gy if there is evidence of macroscopic residue in the surgical bed or mediastinal region.

The use of hypofractionated regimens is not advised due to the risk of increased toxicity. Currently, accelerated fractionation radiotherapy schemes (2 Gy/d, 7 d/wk) are being explored (NCT02189967)[54].

In terms of treatment sequencing, PORT should be administered after completing ChT if the surgical resection is complete (R0); however, in patients with postoperative R1-R2 margins, there is some controversy surrounding the use of concomitant or sequential RT and ChT. As a result, the treatment sequence should be individualized based on the expected tolerance[55,56].

Although several radiotherapy techniques — 3D-CRT, intensity modulated radiation therapy (IMRT), volumetric modulated arc therapy (VMAT), and tomotherapy — all provide optimal dosimetric results in the postoperative context [57], data from prospective studies support the routine use of the IMRT in NSCLC due to lower cardiac doses and a lower risk of severe pneumonitis.

FUTURE LINES OF RESEARCH IN PORT

At present, there is broad consensus among radiation oncologists that the current level of evidence is insufficient to recommend PORT for all patients with stage III pN2 NSCLC, which is mainly attributable to the heterogeneous characteristics of patients with pN2 disease and treatment-related cardiopulmonary toxicity, which remains high despite efforts to reduce it.

In terms of the lack of homogeneity, it is evident that TNM staging in patients with pN2 NSCLC does not provide sufficient information to indicate or not adjuvant therapy. Consequently, it is essential to explore and evaluate new clinical, pathological, and molecular factors to better differentiate between different risk subpopulations, which would then allow us to tailor the treatment indication based on

Table 3 Registered active studies related to postoperative radiotherapy

NCT	Title	Study type
NCT02977169	To Evaluate the Role of Postoperative Radiotherapy in Patients With IIIA(N2) Non-Small Cell Lung Cancer	Interventional
NCT02974426	To Evaluate the Optimal Timing of Postoperative Radiotherapy in Patients With IIIA(N2) Non-Small Cell Lung Cancer	Interventional
NCT04073745	Single Fraction Stereotactic Body Radiation Therapy After Surgery in Treating Patients with Non-small Cell Lung Cancer	Interventional
NCT03006575	Study of Split-course Chemoradiotherapy for Postoperative Locoregional Recurrence of Non-small Cell Lung Cancer	Interventional
NCT02555592	Strategy of Surgical Resection with Adjuvant Therapy for IIIA NSCLC and N2 Disease Only in Subaortic or Paraaortic Level	Observational
NCT02189967	Postoperative Radiotherapy of Non-small Cell Lung Cancer: Accelerated *vs* Conventional Fractionation	Interventional
NCT00880971	Postoperative Radiotherapy for Patients with IIIA (N2) Non-small Cell Lung Cancer	Interventional
NCT01112631	Prospective Study of Quality of Life in Non-small Cell Lung Cancer (NSCLC) Patients Treated With/Without Postoperative Radiotherapy	Observational

the patient's unique characteristics.

It is important to note that most of the prognostic factors identified to date have been derived from data obtained in large retrospective series or epidemiological records. Clearly, due to the important methodological limitations of those studies, it is difficult to extrapolate the findings of those studies into routine clinical practice without stronger supporting data. In this regard, new studies with more robust methodological designs are needed to obtain a higher level of evidence. Table 3 lists the main trials currently underway to evaluate PORT in NSCLC.

The studies performed to date have consistently found an association between PORT and a higher risk of cardiopulmonary morbidity and mortality, a finding that undermines the clinical benefits of this treatment. However, some studies have shown that IMRT is superior to 3D-CRT in NSCLC in terms of dosimetry and survival outcomes[58]. Heavy particle therapy seems to show certain dosimetric advantages *vs* IMRT in terms of protection of OARs, and could significantly reduce cardiopulmonary toxicity, although prospective studies confirming this clinical benefit are not yet available[59].

For all the reasons described above, it is evident that only advanced radiotherapy techniques, such as VMAT or IMRT, which allow for better dose conformity, should be used for the treatment of NSCLC. In addition, these techniques should be used in all future clinical trials of PORT to better determine the true value of PORT in patients with NSCLC.

CONCLUSION

In patients with stage pN2 disease, current evidence suggests that the treatment decision should be evaluated on a case-by-case basis by a multidisciplinary team to determine whether the patient is likely to benefit from PORT. The treatment decision should consider several key clinical features, such as the volume of nodal mediastinal tumor burden, physical condition (performance status) and individual cardiopulmonary risk, but another technological issues, like availability to modern functional imaging devices or high dosimetric conformation radiotherapy (IGRT or VMAT), may be critical for a correct indication.

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REVIEW

Relationship between Th17 immune response and cancer

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Abstract

Cancer is the second leading cause of death worldwide and epidemiological projections predict growing cancer mortality rates in the next decades. Cancer has a close relationship with the immune system and, although Th17 cells are known to play roles in the immune response against microorganisms and in autoimmunity, studies have emphasized their roles in cancer pathogenesis. The Th17 immune response profile is involved in several types of cancer including urogenital, respiratory, gastrointestinal, and skin cancers. This type of immune response exerts pro and antitumor functions through several mechanisms, depending on the context of each tumor, including the protumor angiogenesis and exhaustion of T cells and the antitumor recruitment of T cells and neutrophils to the tumor microenvironment. Among other factors, the paradoxical behavior of Th17 cells in this setting has been attributed to its plasticity potential, which makes possible their conversion into other types of T cells such as Th17/Treg and Th17/Th1 cells. Interleukin (IL)-17 stands out among Th17-related cytokines since it modulates pathways and interacts with other cell profiles in the tumor microenvironment, which allow Th17 cells to prevail in tumors. Moreover, the IL-17 is able to mediate pro and antitumor processes that influence the development and progression of various cancers, being associated with variable clinical outcomes. The understanding of the relationship between the Th17 immune response and cancer as well as the singularities of carcinogenic processes in each type of tumor

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is crucial for the identification of new therapeutic targets.

Key Words: Cancer; Immune response; Th17 cells; Interleukin-17; Tumor microenvironment; Pathophysiology

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Core Tip: Cancer is still an important cause of death worldwide. Its development and progression are intimately related to the host immune response. In that context, the Th17 profile plays crucial roles in the pathogenesis of several cancers, promoting antitumor and protumor mechanisms. This study reviews the interactions occurring between Th17 responses and cancer.

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INTRODUCTION

Cancer represents the second leading cause of death worldwide and has been responsible for 8.97 million deaths from 2000 to 2016 according to the World Health Organization (WHO). The five most lethal malignancy types are lung, liver, gastric, breast, and colon cancers. Four out of the five aforementioned cancers are among the 20 main causes of death in the world and epidemiological studies predict a tendency of increasing mortality rates associated with these diseases in the next 40 years[1]. The knowledge on the relationship between cancer and the immune system was limited for a long time due to the tumor capacity of evading immune response through various mechanisms[2]. However, current evidence has emphasized that $CD4^+$ T cells are intimately associated with processes that unleash cancer as well as with the responses against the malignant cells through the detection of foreign antigens. These cells interact with each other and with other immune system components by releasing cytokines that are able to potentialize or suppress mechanisms in the tumor microenvironment, playing protumor and antitumor roles in that context[3].

Th17 cells is a type of T cell and its main related cytokine is the interleukin (IL)-17, which plays roles in the expression of other cytokines, including IL-6, IL-21, IL-22, interferon (IFN)-γ, and IL-10[4]. The Th17 cells are associated mainly with the immune response against bacteria and fungi, and also perform remarkable contributions in the promotion of inflammation and autoimmunity[5-7]. Moreover, the Th17 profile plays controversial roles in the tumor immunity and can be associated both to unfavorable and favorable outcomes[8]. The duality of Th17 cells in the setting of tumor immunity is potentialized by their plasticity, since they are able to switch to a Th1 phenotype, performing antitumor activities, and they are also capable of expressing a Treg phenotype, which can exert pro and antitumor activities, depending on the context of the immune response[9,10]. The Th17 cells have a peculiar relationship with the tumor microenvironment and use various molecular processes such as the induction and recruitment of further Th17 cells to maintain tumor infiltration[11-14]. Moreover, these cells play important roles in the growing and development of cancerous cells[15]. In that context, the mechanisms performed by Th17 cells that favor or impair tumor progression are complex and depend mainly on the type of cancer. These cells induce some mechanisms with a high carcinogenic potential such as angiogenesis, whereas they can also promote the recruitment of immune system cells to the tumor microenvironment and the activation of effector $CD8^+$ T cells, promoting antitumor activities [16].

The understanding of the mechanisms developed by Th17 cells to modulate the tumor microenvironment and to interact with other cells is a way to identify potential therapeutic targets for various types of cancer that are associated with cellular activities related to that immune profile. Those cells interact in interesting manners

with various interleukins and with cancer stem cells (CSCs)[17]. In addition, the relationship between Th17 cells and CSCs can be considered as bidirectional, with mutual modulation mechanisms[18-20]. This paper aims to review the role played by the Th17 response in various types of cancer, describing its presence in the tumor microenvironment and comparing the repercussions related to this immune profile in cancers, and to approach potential therapeutic targets associated with the immune system mechanisms related to Th17 cells.

CHARACTERIZATION OF THE TH17 RESPONSE

$CD4^+$ T cells can play proinflammatory and regulating roles in the immune response. The classic differentiation of $CD4^+$ T cells into two sets of cells with patterns of cytokine secretion and distinct functions, known as Th1 cells and Th2 cells, has changed with the discovery of a new set of cells known as Th17 cells[21]. Th17 cells express the RORγt (Retinoic-acid-receptor-related orphan nuclear receptor gamma), which is a molecular determinant for its polarization through IL-17A expression[22,23]. In rodents, these cells seem to have the same precursor as $Foxp3^+$ Treg cells, since naive $CD4^+$ T cells stimulated only by transforming growth factor (TGF)-β, convert into Treg cells. On the other hand, TGF-β and IL-6 together induce the emergence of Th17 cells. A study using splenocytes from mice found that an environment containing TGF-β predisposed the emergence of ex-Th17 Foxp3 cells, and simultaneous TGF-β and IL-6 stimuli led to enhanced production of IL-17$Foxp3^{++Neg}$ cells. In addition, that study showed that TGF-β, IL-6, and IL-23 together induced an increase in IL-17A release by Th17 cells[24]. IL-23, in its turn, plays the role of maintaining and expanding these cells. In humans, a relationship between Th17 cells and Th1 cells is evident. Naive $CD4^+$ T cells in the presence of IL-23 and IL-1β positively regulate RORγt, T-bet, IL-23R, and IL-12R. When the two aforementioned cytokines are expressed, IL-17 is produced alone or in combination with INF-γ. TGF-β inhibits the development of both Th1 cells and Th2 cells, and is not essential for the development or inhibition of Th17 cells; therefore, it indirectly favors the expansion of the latter[25,26]. The main function of Th17 cells is to contribute to the immune response against extracellular pathogens during infectious processes, but they are also suggested to play an important role in the pathogenesis of autoimmune and inflammatory diseases, as well as in acute graft-versus-host disease[5-7]. Th17 cells activate neutrophils, stimulate the emergence of CXCL chemokines and MUC5AC, the production of MUC5B mucins by bronchial epithelial cells, the expression of beta defensin-2 and CCL20 by lung epithelial cells, and contribute to the migration and activation of macrophages[7].

The IL-17 family is made up of six different cytokines: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F. IL-17 cytokines have proinflammatory properties, are expressed in various parts of the body, and signals by interacting with their transmembrane receptors. Five receptors from the IL-17 family have been identified: IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE. In addition to Th17 Lymphocytes, $CD8^+$ T cells, macrophages, and γδ T cells produce IL-17. These cells also express IL-23R and secrete IL-21 and IL-22. Among all IL-17 cytokines, IL-17F has the highest degree of conservation with the main cytokine in the family, IL-17A[27]. The IL-17 induces neutrophil recruitment and production of proinflammatory mediators, such as IL-1, IL-8, metalloproteinases 1 and 13, and prostaglandin E2[28,29].

Th17 cells can also produce IL-22. This cytokine is a member of the IL-10 family, and the IL-22R and IL-10R2 receptors have been identified as heterodimeric receptors mediating IL-22 signaling. Whereas IL-10R is ubiquitously expressed, IL-22R is restricted to cells harbored in tissues[30]. IL-22 targets epithelial and non-hematopoietic stromal cells and can promote cell proliferation, playing a role in tissue regeneration. In addition, it regulates host defenses on barrier surfaces. However, IL-22 has also been associated with the development of several diseases involving inflammatory mechanisms[31].

The inflammatory effects produced by IL-17 include stimulus for secretion of IL-6 by human fibroblasts and increased expression of the intercellular adhesion molecule-1[32]. In addition, Th17 cells that co-produce IL-21 regulate B cell responses, induce differentiation of plasma cells, and lead to the formation of antibodies[33]. In that context, the role of Th17 cells as key promoters of inflammation in various pathophysiological contexts, including cancer, has been investigated[8]. Of note, Th17 cells have already been identified in various types of human tumors[8], such as melanoma, ovarian cancer, colorectal cancer, and lung cancer[34,35].

Despite the advances in the knowledge about the roles of Th17 cells in complex biological contexts, many interactions involving these cells remain unknown mainly due to their plasticity[35]. The differentiation of T cells is no longer considered as linear and irreversible, since evidence has shown that populations of differentiated $CD4^+$ T cells can alter the spectrum of cytokines produced and, thus, the outcomes they promote[36]. The plasticity of Th17 cells stands out among the other T cells due to its high level of complexity, involving cytokine-dependent and -independent events, and due to the variability of functional phenotypes that can be adopted by the cells [37]. Th17 cells can play roles that are often heterogeneous, depending on the environmental conditions they are found in. Phenotypes similar to Th1 cells are expressed by Th17 cells in inflammatory environments, with a remarkable production of IFN-γ, contributing as an immunological support against fungal and extracellular bacterial infections, but also for intestinal inflammation observed in colitis, when the activity of these cells is not properly controlled[38-40]. The transfer of antigen-specific Th17 cells to a host has shown that Th17 cells can assume the phenotype of a Th2 cell in infections by *Nippostrongilus brasiliensis*[41]. This Th17/Th2 conversion has also been identified in the peripheral blood of patients with asthma, whose Th17/Th2 cells secreted cytokines from both profiles (Il-17, IL-22, IL-4, and IL-5), which evidences the pathogenic potential of these cells in the induction of intense inflammatory infiltrates [42]. In addition to their pro-inflammatory role, Th17 cells are also able to adopt a phenotype similar to that of Treg cells. In fact, both the conversion of Treg cells into Th17 cells and the conversion of Th17 cells into Treg cells are described by previous studies, possibly because both cell types share differentiation characteristics, given the participation of TGF-β in inducing the differentiation of both Treg and Th17 cells[43]. Although TGF-β, IL-6, and IL-23 alone tend to promote IL-17A release by Th17 cells, the presence of Prostalglandins E2 (PGE_2) makes the stimuli with these cytokines result in the conversion of Th17 cells into regulatory IL-$17A^{neg}$Foxp3 cells, which demonstrates the importance of the PGE_2 in the transdifferentiation of Th17 cells along with TGF-β[24]. Although it presents a certain stability, the aforementioned process has been shown to be bidirectional and, in the presence of Th17-polarizing cytokines, Th17-derived Treg cells can reassume their original Th17 cells phenotype (Figure 1) [9]. Moreover, in response to IL-27, Th17 cells acquire a phenotype similar to that observed in TR1 cells *via* activation of the Blimp-1 factor, which results in the secretion of cytokines such as IL-10[44].

RELATIONSHIP BETWEEN TH17 CELLS AND CANCER

Although advances have been achieved in the study of Th17 responses, the knowledge regarding its roles in the immune system is still limited. Moreover, the tumor microenvironment has been broadly studied because it is directly associated with cancer development and progression. The understanding on how the immune system cells behave in the aforementioned microenvironment and how this environment is influenced by those cells make possible a broader comprehension about cancer and the therapeutic possibilities against this disease[45]. The participation of Th17 cells in the different types of cancer is paradoxical, since they can play antitumor and protumor roles[8]. It is believed that the Th17 cells prevail in the tumor microenvironment through some mechanisms, including: (1) The induction of T cells involving the TGF-β 1 and IL-6. Signal transducer and activator of transcription 3 (STAT3)[11]; (2) Recruitment of Th17 cells dependent on various chemokines including CCL20, CCL17, CCL22, MIF, RANTES, and MCP1[12-15]; (3) Conversion from other cell types[15]; and (4) Th17 cell polarization through cytokines such as IL-1β and IL-13 that are produced in the tumor environment by specific myeloid cells[46]. In addition, the tumor microenvironment has antigens and metabolites that are able to suppress $CD4^+$ T cells, which start producing co-inhibitory and less effective molecules[47]. However, the Th17 cells from the tumor microenvironment seem to have an enhanced resistance to dysfunctionality because they present less exhaustion markers than other T cells as well as more CCR7, Lef1, and TCF7 markers[48,49].

An important aspect regarding Th17 cells in cancer settings is the aforementioned plasticity potential through their transformation into Treg cells, which occurs with TCR engagement leading to the expression of Foxp3 and subsequent imunossupressive roles by those cells. Of note, $IL17^+Foxp3^+$ T cells have been associated with the emergence of CSCs and with the inhibition of tumor-specific T $CD8^+$ cells in colorectal cancer. A study showed that $IL17^+Foxp3^+$ T cells induce the expression of markers such as CD133, CD44s, CD166, EpCAM, and ALDH1 in bone marrow-derived mononuclear

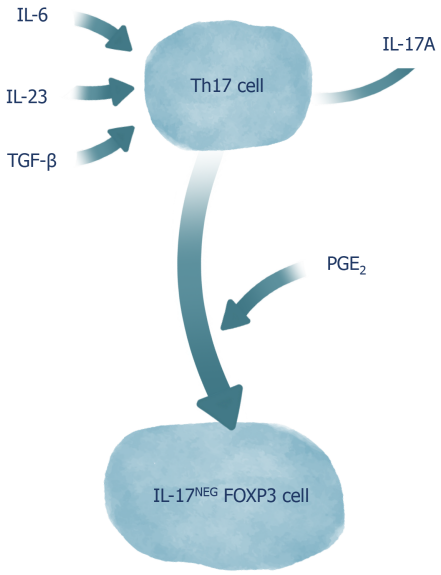


Figure 1 Interleukin-6, interleukin-23 e transforming growth factor-β stimuli for interleukin-17A production and the role of Prostalglandins E2 in Th17 cells transdifferentiation into interleukin-17^{NEG} FOXP3 cell. TGF: Transforming growth factor; IL: Interleukin; PGE_2: Prostalglandins E2.

cells and promoted their conversion into cancer-initiating cells[50,51].

Conversely, the production of IL-6 in mice with melanoma led to the conversion of Treg cells into Th17 cells, which resulted in the promotion of the activation of $CD8^+$ T cells and reduction in tumor growth[52]. A recent study on CpG (ODNs)/CpG 1826 oligodeoxynucleotides demonstrated the potential of these cells in the inhibition of Treg cells and in the stimulation of Th17 cells, extending survival among mice with leukemia[53]. Another study observed that the Treg/Th17 cell ratio was higher among patients with oral squamous cell carcinoma (OSCC) than in controls, suggesting that these cells are involved in the progression of OSCC and have the potential to be used as a prognostic indicator[54]. Considering the importance of the Th17/Treg axis in cancer-related immune response and inflammation, a study assessed the metabolic features involved in that setting. The researchers found that Th17 cells are more dependent on the synthesis of fatty acids than Treg cells, which primarily perform the oxidation of fatty acids to keep their energetic homeostasis. This metabolic differences can make the manipulation of the Th17/Treg axis possible for new therapeutic alternatives against neoplasms[55].

Interestingly, the plasticity of Th17 cells has also been observed with their conversion into Th1 Lymphocytes, exerting antitumor effects[10,56]. It is not well known if that transformation occurs inside the tumor microenvironment or if Th17/Th1 cells are recruited to the tumor microenvironment[57,58]. Muranski *et al*[48] reported that the Th17 cells polarization leads to the production of Th1 cells-related molecules such as INF-γ and T-bet, which are associated with remarkable antitumor activities[48].

The mechanisms taking place in tumor-related Th17 responses are various and depend on the type of cancer. A study observed that the transference of Th17 cells implied in the recruitment of $CD4^+$ T cells, $CD8^+$ T cells, and DCs for the tumor microenvironment. Additionally, when in contact with tumor antigens, Th17 cells acquired dendritic cell MCHI-peptide complexes and, through MHCI-TCR interaction and IL-2 release, there was an activation of $CD8^+$ T cells, reinforcing the role of Th17 cells in tumor immunity[16]. Studies have verified that IL-17 may indirectly potentialize the functions of cytotoxic T lymphocytes by stimulating the expression of IL-6 and IL-12, leading to antitumor effects[59]. The Il-17 acts in the recruitment and expansion of neutrophils that are essential to the destruction of tumor cells as well as contributes to the expression of various proinflammatory molecules including IL-1β, IL-6, tumor necrosis factor (TNF)-α, PGE_2, CXCL1, CXCL5, CXCL8, and GMCSF[60-65]. On the other hand, Wang *et al*[54] demonstrated that Th17 cells play protumor roles since IL-17 induces the expression of IL-6, which is responsible for activating oncogenic signal transducers and STAT3. The STAT3 aids in the promotion of tumor growth through the regulation of pro-angiogenic genes[66]. Differently from the IL-17 dualism, the IL-22 has shown to be a protumor interleukin that, through STAT3, is involved in the development of tumor cells[67-69].

The Th17 cytokines, especially IL-17, play important roles in the promotion of tumor angiogenesis[70]. Studies have described that the IL-17 induces the expression of vascular endothelial growth factor (VEGF), and that cytokine seems to be associated with a higher tumor vascular density[71]. In addition, the Il-17 induced the expression of angionenic chemokines including CXCL-1, CXCL-5, CXCL-6, and CXCL-8, leading to an increased angiogenic potential among immunocompromised rats with non-small cell lung cancer (NSCLC)[72]. Another study described that IL-17 is involved in the activation of angiogenic genes through the IL-6/STAT3 pathway[73]. The IL-22 has also been positively correlated with angiogenesis. A study suggested that it is involved in the proliferation, survival, and migration of endothelial cells. Moreover, that investigation described that IL-22 leads to vascular growth in animals[74]. Finally, a systematic review highlighted that, although there is a paradox in the behavior of the Th17 response in the different types of cancer, the expression of Th17 cells is often associated with better prognosis whereas the IL-17 is related to cancer progression[75].

Studies have described the IL-27 potential as a Th17 response inhibitor. This process occurs through the downregulation of RORγt[17]. The IL-27 is involved in the suppression of protumor cytokines such as IL-23 and IL-17[76]. A recent study described the existence of an inverse relationship between IL-27 and IL-17 as well as between IL-27 and IL-6. In patients with gastric cancer, studies have observed high concentrations of Th17 cells-related cytokines including IL-1β, IL-6, IL-17A, IL-23, and TGF-β and it is believed that this phenomenon occurs due to the low or null IL-27 Levels, since IL-27 inhibits RORγt and IL-6. This relationship has been observed in other cancers as well[77]. Additionally, another study observed that the IL-27 performed an antitumor activity through the IL-17 inhibition *via* RORγt among patients with small-cell lung cancer[78]. Of note, these findings suggest a therapeutic potential of the IL-27 through the inhibition of protumor Th17 cells-related mechanisms.

Interestingly, the cancerous cells inside individual tumors frequently exist in various phenotypic states. CSCs are a subpopulation of cells present in several types of cancer and that have self-renewal ability and tumorigenicity when transplanted to an animal host. Evidence on CSCs have aimed at developing a promising approach for the improvement of antitumor therapies. The CSCs are closely related to Th17 cells-related cytokines and other components in the tumor microenvironment, and they play crucial roles in the tumor progression and metastasis[79,80]. These cells can promote the differentiation of $CD4^+$ T cells into Th17 cells through the release of soluble mediators and cell-to-cell contact[18,19]. Moreover, a study observed that the interaction between stem cells and $CD4^+$ T cells can lead to the transformation of remaining Th17 cells through the activity of STAT3[81]. On the other hand, the IL-17 has shown to participate in the maintenance of CSCs through the action of the IL-17A receptor and the capacity of activating these cells in their quiescent state, configuring a more aggressive protumor behavior for the CSCs[20]. The recurrence of tumors after a primary treatment is still very frequent, and studies have supported that a successfull cancer therapy might be related to the elimination of CSCs[82]. In that context, the understanding of the relationship between these cells and the Th17 response in the various types of tumor can lead to new therapeutic possibilities[18].

ASSOCIATION BETWEEN TH17 RESPONSE AND DIFFERENT TYPES OF CANCER

Urogenital system cancers

Ovarian cancer: Ovarian cancer ranks fifth in cancer deaths among women[83]. This cancer usually has a late and advanced diagnosis causing resistance to treatment[84]. Studies have emphasized the involvement of the immune system in the process of development and progression of ovarian cancer, including the interactions of immune cells in the ovarian tumor microenvironment[85,86]. The expression of IL-23 is increased and positively correlated with IL-17 in immunohistochemical analyses of ovarian cancers[87]. Studies showed that IL-17 and IL-6 induce STAT3 phosphorylation, leading to the production of programmed death-ligand 1 (PD-L1), which is related to the inhibition of antitumor T cells[88-90]. Additionally, T lymphocytes and antigen presenting cells can stimulate the secretion of proinflammatory interleukins such as IL-1, IL-6, and IL-23, providing favorable means for the differentiation and expansion of Th17 cells[91]. In addition, ovarian tumor cell lines secrete IL-6, IL-8, IP-10, MCP-1, and VEGF in large amounts[91]. The IL-17 stimulates tumor progression due to proangiogenic effects and increased percentage of $CD4^+$/IL-17 T lymphocytes

among cells that infiltrate ovarian cancer[92,84]. The chemokine CXCL12 (SDF-1α) participates in the induction of angiogenesis verified in several types of tumors along with VEGF[93]. Curiel *et al*[94] demonstrated that ovarian cancer patients had increased levels of proangiogenic cytokines (IL-8 and TNF-α) that stimulate the development of new tumor blood vessels[94]. This abnormal vasculature allows the tumor to spread more effectively, facilitates the escape of immune surveillance, and impairs the action of antineoplastic agents, limiting the beneficial effects of these drugs [84,85]. Notably, IL-17A and gdT cells recruit small peripheral peritoneal macrophages that selectively secrete IL-17A receptors that trigger tumor growth and angiogenesis [95].

Another study reported that there is an inverse relationship between Treg and Th17 cells observed in ovarian cancer and this difference leads to an antitumor response, which significantly influences survival, and the presence of these polyfunctional Th17 cells is statistically related to more favorable clinical outcomes[96]. In contrast, the significant presence of regulatory T cells is associated with worse survival[97]. Studies suggest that the responses of T cells directed to ovarian cancer may be related to the expansion of Th17 cells and may neutralize the suppression of these cells through the activity of Treg cells[97,98]. Moreover, the decrease in Th17 Lymphocytes and the increase in Treg cells was related to an increased level of TGF-β and this, in turn, is associated with metastatic processes resulting in poorer clinical prognosis[99,100]. Additionally, in vivo studies observed that a high Treg cells/Th17 cells ratio seems to predispose tumor progression because this ratio has shown to be significantly higher in epithelial ovarian cancer and peritoneal metastasis than in benign ovarian tumors and benign peritoneum[101]. It is believed that the presence of tumor-associated macrophages may induce this imbalance[102]. M2 macrophages have been shown to modulate the tumor microenvironment and to promote a relative deviation to the Treg immune profile through the release of exosomes carrying miRNAs that are often overexpressed in some types of cancer, such as ovarian cancer[103,104].

Th17 cells in ovary cancer secrete IL-21, which, along with TGF-β, can interfere in the differentiation of T cells into Th17 Lymphocytes and stimulate the transformation of Th17 cells into Treg cells. In addition, there is a regulation in the distribution of Th17 and Treg cells *via* CCR6 chemokine receptors to direct Th17 cells to specific sites [105,106]. In this sense, some therapeutic methods have been developed aiming at increasing the patients' survival as well as at reducing the recurrence rates. Among these methods, stand out immunotherapy approaches based on therapeutic vaccines that stimulate the expansion of specific T cells in patients with ovarian cancer, using the alpha folate receptor (Fra) as the vaccine target antigen, since this receptor is overexpressed in patients with high-grade serous ovarian cancer[97,107]. The vaccine induces stimulation of Fra-specific INF-γ^+ and IL-17^+ T cells[97]. Furthermore, a study showed that the Th17 profile and IFN-γ induce the production of CXCL9 and CXCL10 that promote the migration of effector cells to ovarian tumors[98,108]. The use of vaccines that stimulate the expression of Th17 cells specifically in an ovarian tumor is associated with a reduction in recurrence rates, as well as in the improvement of survival rates[97,98].

Prostate cancer: Prostate cancer is the most prevalent type of cancer among the male population[109]. Chronic inflammation has been identified as a factor that is associated with the pathogenesis of various types of cancer, including prostate cancer[109,110]. In that context, IL-17 plays an important role in the inflammatory process related to the development and progression of that cancer[111]. Studies have shown that blocking IL-17 in mice inhibits the development of prostate cancer[112]. The imbalance in the proportion of $CD4^+$ and IL-17^+ cells and $CD4^+$ $Foxp3^+$ T cells, responsible for regulating Treg cells in the tumor microenvironment, may lead to worsening of the inflammatory process and promote carcinogenesis[113,114]. Treg cells are involved in the suppression of antitumor immune responses[115]. Blocking PD-1 can promote antitumor activity by balancing Th1/Th2 responses and stimulating Th17 cells, as well as inhibiting Treg cells and stimulating the Th17 response[116]. In that context, the use of vaccines that inhibit the action of programmed death 1 (PD-1)/PD-L1, which make up an immunological checkpoint, has demonstrated promising results[117,118]. In prostatic tumors, IL-17 has been shown to attract M1 and M2 macrophages. M1 macrophages inhibit tumor growth, whereas the M2 cells promote tumor growth[119-121].

Respiratory tract cancers

NSCLC and small cell lung cancer: Chronic inflammation is a crucial factor in the pathogenesis of different types of cancer, including lung cancer, which has proved to

be a major public health problem affecting 1.8 million patients each year[122]. Lung cancer is the leading cause of cancer-related deaths in recent decades and can be divided into two types: Small cell lung cancer and NSCLC[123]. In addition to Th17 cells and the association with tumor survival, chemokines and their receptors related to T cell migration were examined in NSCLC cases. The high expression of CCR6 was associated with shorter disease-free survival[124]. Likewise, CCL20, a chemokine known to interact with CCR6, was elevated in the tumor compared to tumor-free lung tissue. Thus, these results suggest that CCL20/CCR6 may facilitate the infiltration of Th17 cells in the NSCLC and promote tumor progression[125]. A regulatory role for Th17 cells in the tumor microenvironment of NSCLC was found to modulate the differentiation and activation of various subsets of local T cells[126]. IL-17A is associated with the concentration of VEGF in patients with NSCLC, suggesting that IL-17A may promote angiogenesis in that tumor[127]. In addition, patients with high levels of IL-17A demonstrated shorter survival compared to those with low expression of the cytokine[128]. Studies suggest that IL-17A/Th17 cells may play a pro-tumorigenic role, as an increased number of Th17 cells are found in lung cancer[129]. Th17 cells can be generated under oncogene activation or inhibition of tumor suppressors in human and murine models[130,131]. Oncogenic NSCLC models have shown a predominant pro-tumorigenic role of IL-17A[130,61], while downregulation of IL-17A in a tumor suppressor NSCLC model has been associated with antitumor activities[131].

Studies also demonstrated that IL-17A deficiency or blockade leads to the suppression of lung metastasis in experimental tumor models. This suggests that the key cytokine IL-17 produced by lung $CD4^+$ Th17 cells plays an important role in cancer regulation[132]. In addition, anti-IL-17A treatment in pulmonary adenocarcinoma modifies cytokine responses by lung $CD4^+$ T cells and induces production of TNF and IFN-γ by Th1 cells at the tumor site, leading to improved antitumor immune responses and suppression of tumor growth[133]. Other studies assessed the role of IL-6, a cytokine produced by Th17 cells, in a murine model of lung adenocarcinoma and human tumors, showing that IL-6 inhibits regulatory T cells and induces Th17 cells. In vivo treatment with anti-IL-17A antibodies reduced the production of IL-6 in the airways[132]. Thus, anti-IL-17A-mediated regulatory T responses can induce increased anti-tumor immune responses[134]. The main transcription factors of Foxp3 regulatory T cells, as well as the main transcription factors of Th17 in human cells, were increased in lung tumor tissues, resulting in a parallel local expansion of Th17 and Treg responses. These findings suggest a potential direct relationship between both T cell lines in lung cancer[135]. T-cell immunoglobulin-3 (TIM-3) expressed in Th1, Th17, and CD8 T cells, but not in Th2 cells, has already been described as a critical component of cell-mediated immunity against cancer[136]. Recent studies have supported an important role in the exhaustion of TIM-3 T cells in lung cancer[137]. TIM-3 as well as PD-1, another T cell exhaustion marker, are co-expressed in TCD8 in mice with lung tumors, exhibiting depleted phenotype as defined by the failure to proliferate and produce IL-2, TNF, and IFN-γ[137]. Blocking the TIM-3 and PD-1 pathways is more effective in controlling tumor growth than targeting either pathway alone, suggesting that these two pathways work synergistically in establishing T-cell exhaustion[138].

Gastrointestinal tract cancers

Gastric cancer: Gastric cancer is the fifth most common and the third most lethal malignancy around the world. The development of this cancer is mainly linked to factors such as the chronic inflammation induced by *Helicobacter pylori* infection and age[139]. It is well established that the immune response of the host infected by *Helicobacter pylori* leads to the activation of the IL-23 pathway, which induces the differentiation of $CD4^+$ naive T cells into Th17 cells. This immunological pathway is called the IL-23/IL-17 axis[140]. The IL-17 acts in the endothelium, monocytes, and gastric epithelial cells producing TNF-α, IL-1, IL-6, and IL-8 that stimulate the recruitment of neutrophils to the inflammatory site[140,141]. In an interesting study, a significant increase in the levels of IL-17 was observed in the serum of patients with gastric cancer. In addition, this increase in IL-17 expression was associated with a high density of microvessels, which assist in the development of the tumor[142]. An important study also found high levels of plasma IL-17 in a patient with gastric cancer and, interestingly, the results showed an increase in the expression of IL-17 and RORγt in the cancer tissue. In addition, a 26-fold increase in IL-17 expression was observed among patients who had metastasized[143].

Moreover, a study found high levels of IL-6, TGF-β1, FoxP3, and IL-17 expression in gastric cancer patients, highlighting the importance of the Th17/Treg axis in this neoplasm. IL17 and IL-6 have been associated with tumor progression, and Foxp3 and TGF-β1 were mainly expressed in patients with advanced gastric cancer. These findings suggest that these molecules play a role in the tumor immune response evasion and cancer progression[144].

The induction of Th17 cell expression is very important in the pathophysiology of gastric cancer. Moreover, it is well described that IL-1, IL-6, IL-21, IL-23, and TGF-β induce the differentiation of T naïve cells into Th17 cells[145]. In this sense, Su *et al* [143] also observed increased levels of TGF-β and IL-21 in gastric cancer tissues[143], positively regulating Th17 cells and, consequently, IL-17 levels. Some studies suggest that treatment with anti-IL-17A monoclonal antibodies such as Secukinumab and Ixekizumab may be beneficial in gastric cancer therapy[146]. An experimental study with rats, which had tumor growth stimulated by injection of gastric cancer cells of the YTN16 type, showed an expressive regression of the tumor with a complete elimination of the cancer in 8 of 10 mice using a combination of anti-IL-17A and anti-PD-1 monoclonal antibodies[147]. Despite being experimental results, they point to new paths regarding the treatment of gastric cancer with immunotherapy.

Pancreatic cancer: Pancreatic cancer is the 14th most common cancer and the 7th that kills the most in the world[148]. Obesity, type 2 diabetes, and smoking are the main risk factors for the development of this cancer[149]. Th17 cells are important in tumor-associated inflammation, stimulating migration, invasion, and induction of angiogenic factors[150]. A recent study observed that IL-17 stimulates an important mediator of pancreatitis (REG3-β) in pancreatic cells and can activate the gp130-JAK2-STAT3-dependent signaling pathway, which results in a greater acinar-ductal metaplasia and in the development of lesions of early pancreatic intraepithelial neoplasia[151]. An interesting study pointed to a similarity between the IL-17A-IL-17RA pathway and the IL-17B-IL-17RB pathway in tumor malignancy. It was observed that IL17-B increased the expression of IL-8, Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and activating protein-1 (AP-1), which aid the tumor invasion in the pancreatic tissue and recruit neutrophils, lymphocytes, and endothelial cells[152]. Although advances have been achieved, the roles played by Th17 cells in the development of pancreatic cancer are not well understood. Previous investigations in mice suggest how this pathway works. However, the mechanism in humans needs further studies. Some studies have observed increased levels of Th17 cells in patients with pancreatic ductal adenocarcinoma, supporting theories about the role of this profile in the immune response involved in this cancer. He *et al*[153] reported that the frequency of these cells was higher in pancreatic tumor tissues when compared to other tissues of the organ (P = 0.031). Serum levels of IL-17 and IL-23 were significantly increased in pancreatic cancer patients when compared to healthy patients (P = 0.02)[153]. IL-17 blockade demonstrated an inhibition in neutrophil recruitment and increased activation of CD8$^+$ T cells in the environment close to the tumor. In addition, a synergy was observed between the inhibition of IL-17 and PD-1 in the therapy against that cancer[154]. Interestingly, a study that aimed at understanding the Th17/Treg axis and its implications in pancreatic cancer found that the balance between these immune response profiles was altered in the peripheral blood of patients with this malignancy, with an important deviation to the Treg immune profile. Furthermore, it was observed that this relationship becomes even more accentuated as the disease progresses, supporting the hypothesis that Treg cells might impair the antitumor immune response and contribute to the tumorigenesis in pancreatic cancer[155]. Despite these results, studies for the clinical use of these agents are still scarce.

Colorectal cancer: Colorectal cancer is the third most common cancer and the fourth with the highest mortality in the world[156]. The main risk factors for the development of this cancer are age, genetics, obesity, type 2 diabetes, and inflammatory bowel disease[157]. Possibly, IL-17A acts to increase IL-6 and VEGF, which are important in carcinogenesis and pro-angiogenic, respectively[158]. Other studies indicate that Th17 cells stimulate immunosuppressive factors such as TGF-β, CXCR3, chemokine receptor CC 6 (CCR6), and IL-6. In addition, they decrease the anti-tumor activities of CD8$^+$ T cells[159]. An interesting Chinese study observed the positive regulation of IL-17 in the progression of the adenoma-carcinoma sequence, with the levels of this cytokine being higher in cancer patients[160]. Although the pathogenesis of colorectal cancer has different pathways, in fact, IL-17 has an important role in the immune response and in the development of this cancer. The possible treatments that have been studied in this

setting involve blocking IL-23, IL17, IL-17R, and RORγt nuclear receptor antagonists, which can inhibit the differentiation of Th17 cells[159]. However, clinical trials with these agents have not been developed and further studies are needed to understand the effectiveness of these drugs in patients at different stages of this disease.

Liver cancer: Liver cancer is the fifth and ninth most common cancer in men and women, respectively, and has a high mortality rate around the world[161]. Infection with hepatitis B and C viruses, alcoholic liver disease, and, possibly, non-alcoholic fatty liver disease are the main risk factors for the development of hepatocellular carcinoma[162]. A recent Chinese study found that IL-17 has a direct effect over hepatocellular carcinoma with the induction of IL-6/JAK2/STAT3 by activating the AKT pathway. This pathway positively regulated IL-8, matrix metalloproteinases 2 (MMP2), and VEGF, and neutrophil recruitment, neoangiogenesis, and tumor growth were observed *in vivo*[163]. Another study also indicated the influence of the CCL20/CCR6/Th17 cells pathway in promoting vascular invasion and metastasis [164]. A study identified that the high intramural expression of IL-17 and IL-17E were predictors of a worse survival prognosis (P = 0.016; P < 0.001)[165]. It has been well described that IL-17 is important in the development and prognosis of hepatocellular carcinoma. However, there are not many studies on immunological therapy, and the concomitant use of IL-17 inhibitors with conventional treatments, may be a promising alternative to be explored by new studies.

Skin cancers

Non-melanoma skin cancer: According to the WHO, skin cancer has increased over the past 20 years. The global estimate is that there will be 2 to 3 million cases of non-melanoma cancer and 132000 melanoma cancers annually[166]. Previous animal model studies using the chemical inductors dimethylbenzanthracene (DMBA) and 12-O-tetradecanoylforbol-13-acetate (TPA) that promote the development of inflammation-associated skin cancer, have demonstrated that IL-17R-deficient mice are resistant to DMBA/TPA and that the depletion of this cytokine increases the immune control performed by $CD8^+$ T cells and inhibits the promotion of inflammation in the skin tumor. It has also been found that TPA-induced inflammation increases the susceptibility of tumor growth and the development of tumor-specific IL-17-producing T cells and that IL-17 blockade can inhibit the progression of existing skin tumors stimulated by chemical reagents and cancel the induced inflammation that contributes to tumor growth[167]. In another study using the same experimental model, in addition to looking at the importance of IL-17-produced by $CD4^+$ T cells in skin tumorigenesis, the researchers revealed an important regulatory role in the IL-17-STAT3 pathway in tumor development, verifying that IL-17 induces the oncogenic activity of STAT3 and promotes the proliferation of epidermal cells and hyperplasia. On the other hand the decrease in IL-17 reduced the activation of STAT3 and the unleashing of its protumor mechanisms[168]. In a research with samples of patients diagnosed with basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) of the skin, the role of the cytokines IL-17 and IL-22 in the progression of both types of cancer was evaluated. The results showed that the tumor microenvironment in both carcinomas is enriched with $IL\text{-}22^+$ and $IL\text{-}17^+$ T cells. The IL-17, alone or along with TNF-α, was able to induce the production of IL-6 and IL-8, important for tumor progression in the analysis of SCC CAL27 cells. Another important finding is that IL-17 positively regulated NF-κB signaling, while IL-22 activated the STAT3 pathway and the anti-apoptotic AKT protein in both cell lines. Corroborating the *in vitro* findings, experiments with mice that received CAL27 also demonstrated that IL-17 and IL-22 increased the size of the tumor[169]. Another study with human BCC biopsies characterized by a moderate-to-severe inflammatory infiltrate evaluated the expression of the cytokines IFN-γ, IL-23, IL-17, and IL-22 and their expression during treatment with the drug imiquimod (IMQ) and with photodynamic therapy (PDT). The results showed high expression of all cytokines in cancer, and they were related to the severity of the inflammatory infiltrate. It was also possible to observe a correlation between IFN-γ and IL-17 expression, and both cytokines were expressed by $CD4^+$ and $CD8^+$ T cells. In addition, there was an increase in all cytokines in response to IMQ/PDT treatment[170].

Melanoma skin cancer: Melanoma is the 19th most frequent neoplasm in the world with an incidence rate of 3.3 *per* 100 thousand people[171]. Malignant melanoma is the type of skin cancer with the worst prognosis, having a high probability of spreading metastases when in advanced stage[172]. IL-17 promotes tumor growth by facilitating angiogenesis and the exit of tumor cells from their primary focus[172,173]. The expression of IL-17A represents an important target in the study of the escape of

tumor cells from the immune system[171]. A research conducted in 2010 points to IL-17 as a useful biomarker for early diagnosis of melanoma in mucosae[174]. It is believed that the unregulated tissue inflammatory process may contribute to tumor expansion and metastasization[175-177]. A study analyzed the effect of IL-17 on the growth of melanoma, and wild type mice and IL-17-/-were inoculated with a B16 melanoma cell lineage. The results showed that melanoma growth was significantly inhibited in IL-17-/- mice compared with wild type mice. In this same study, the Hmgb1 (High-mobility group box 1) molecule and its receptor RAGE, which is associated with inflammation and cellular injury, were also analyzed. The growth of the B16 cell lineage was inhibited and the expression of IL-23 and IL-17 was significantly reduced in RAGE-/-mice, indicating that the Hmgb1-RAGE route contributes to the IL-17 expression dependent on the production of IL-23, and promotes tumor growth[176]. A previous study demonstrates that the production of IL-17 by B16 melanoma strains induces the production of IL-6 that activates the nuclear transcription factor STAT3, which acts activating the transcription of several genes related to cell proliferation and to an increase in the expression of VEGF, MMP9, and prostaglandins E1 and E2. Moreover, STAT3 increases the expression of anti-apoptotic genes[178]. Thus, it can be concluded that IL-17 promotes the growth of melanoma B16, while the blocking of IL-17 inhibits the growth of the tumor. On the other hand, Th17 cells might play a protumor role when they are converted into a hybrid phenotype expressing markers that characterize both this cell subtype and Treg cells. This conversion also occurs *via* the secretion of TGF-β and retinoic acid in the presence of suppressor cells derived from tumor-infiltrating myeloid cells. These findings reinforce that Th17 cells might play conflicting effects on melanoma, also contributing for its pathogenesis[179]. These studies support the involvement of Th17 cells and interleukins produced by this population of T lymphocytes, highlighting the cytokine IL-17, in the development and progression of skin cancer. In addition, there is also the possibility of new therapeutic approaches targeting this immune response profile. Finally, further studies are needed to better understand the relationship between Th17 responses and skin cancers.

Other types of cancer

Cervical cancer: Cervical cancer is the second leading cause of cancer death in young women worldwide. Virtually all cervical cancers begin with infection with high-risk human papillomavirus (HPV)[180]. Most HPV infections are eliminated naturally as a result of humoral and cellular immune responses[181]. However, the persistence of this infection induces an inflammatory response, which seems to contribute to tumor growth and disease progression, instead of inducing an effective immune response [182]. This response is partially induced by tumor cells that regulate the immune response negatively through the expression of human leukocyte antigen, which produces immunosuppressive cytokines, such as IL-10 and TGF-β, and attracts regulatory T cells[183].

A large number of Th17 cells was a factor that improved prognosis and survival in this type of cancer, suggesting that Th17 cells play important antitumor responses in cervical cancer settings[183]. Evidence emphasizes that the infiltrate of Th17 cells in the cervical tumor had an activated phenotype with increased expression of CCR6, the receptor for the CCL20 chemokine. It corroborates the hypothesis that the Th17/Treg axis imbalance may be involved in the promotion and progression of cervical cancer [184]. A recent study suggested that the imbalance between Th1/Th2 and Th17/Treg cells was related to the stage of cervical cancer, tumor size, metastasis, and vasoinvasion. The findings demonstrated that the peripheral immune cell levels reflect the patient's condition[185]. Although some questions can already be answered, further studies on the involvement of the Th17 response to cervical cancer are still needed.

Lymphoma: Every year, approximately 500000 people are diagnosed with non-Hodgkin's lymphoma and 80000 with Hodgkin's lymphoma, the most common cancers among the 90 lymphoma subtypes[186]. In a study conducted in China, a significantly decreased frequency of Th17 cells was observed in the peripheral blood of patients with non-Hodgkin's B-cell lymphoma compared to healthy individuals, along with an increase in Th1 cells[187]. This activity may be associated with the patient's response to treatment and the different stages of the disease[188]. Another study revealed that the number of Th17 cells in lymphomas is influenced by the amount of mast cells and granulocytes. The IL-6 expression by these cells contributes to the establishment of a pro-inflammatory environment for Th17 cells, favoring CXCL-13 production and its interaction with CXCR3 and CXCR5 receptors expressed in mast

cells[189]. It has been shown that drugs that block the IL-23/IL-17 axis, which are already available for the treatment of certain autoimmune diseases, can increase the therapeutic impact against classic Hodgkin's lymphoma[190,191]. Moreover, a recent study demonstrated that the prognostic implication of Th17 cells depends on the type of treatment employed, since the Th17 signature did not represent a negative prognosis in the treatment with the medication Lenalidomide in non-follicular lymphoma[192,193].

Breast cancer: Breast cancer is one of the main causes of death among women[194]. This type of cancer is a heterogeneous disease with different patterns of tumor infiltrating lymphocytes, depending on the molecular subtype and other factors of the tumor microenvironment that are important for prognosis and predictive for treatment [195]. Studies point to a relevant infiltrate, characterized mainly by $Foxp3^+$ cells and high levels of IL-6, in addition to revealing a high infiltration of IL-17-producing cells and a low amount of $CD8^+$ cells, suggesting that Th17 cells participate in an effective immune response to eliminate the tumor in patients with breast cancer[196,197]. IL-6 is expressed in breast cancer patients, and its levels are positively associated with the number of Th17 cells. In the breast cancer microenvironment, IL-6 enhances the differentiation and expansion of Th17 cells[198]. Another study showed that Th17 cells positively regulate the production of CXCL1 during the progression of breast cancer. CXCL1, which is produced by breast cancer cells, might promote the growth and development of cancer[199]. In a recent study carried out in China, the involvement of high salt intake was evidenced as a factor that accelerated the growth of breast cancer in addition to increasing Th17 cells circulation in mice. It was also demonstrated in an *in vitro* study that the elevation of Th17 cells was reversed with the application of 1.25 Vitamin D3, inhibiting the differentiation of these cells ($P < 0.001$)[200]. Although some studies point to the important protective role of Th17 cells, further investigation is needed in that context[201].

Bone-related cancers: Studies evaluating the relationship between Th17/Treg axis and bone marrow cancer concluded that deregulations in this axis leading to immune tolerance or impaired immune response might contribute to bone tumorigenesis. In that context, a study evaluating peripheral blood mononuclear cells and bone-marrow mononuclear cells from patients with multiple myeloma and healthy controls observed an enhanced expression of Th17 cell-related cytokines in the affected individuals. Moreover, that study demonstrated that the IL-17 has the potential to promote the growth of myeloma cells and colony formation through the activation of IL-17 receptors as well as to inhibit the Th1 immune system profile along with the IL-22[202]. Otherwise, studies have observed an increased number of Treg cells in patients with acute myelogenous leukemia (AML) compared to controls, which suggests that the Treg immune profile might contribute for an improper immune response against the malignancy and to a consequent progression of the AML[203, 204]. Furthermore, a study investigating the role of IL-22 produced by Th17 and Th22 cells in osteosarcoma demonstrated that its levels were enhanced in osteosarcoma cells and that it stimulates the proliferation and invasion of tumor cells *via* STAT3 signaling [205]. Finally, the aforementioned studies suggest that the understanding of the role of the Th17/Treg axis is essential for the study of bone-related cancers and should be explored as a potential therapeutic target in the treatment of those malignancies. Interestingly, vitamin D3 can act therapeutically over Th17 cells, reducing IL-17A and IFN-γ levels in rheumatoid arthritis. Therefore, vitamin D3 might could be used for the development of a therapeutic approach against bone-related cancers through the modulation of the Th17 immune profile (Figure 2)[206].

CONCLUSION

The Th17 response is intimately linked to the development of cancers and, in the last few years, the knowledge on the role of Th17 cells in the tumor microenvironment have significantly increased. However, much still has to be done in order to achieve a broader understanding on this issue. The IL-17 stands out among the Th17 cells-related inflammatory cytokines, being involved mainly in processes that promote tumorigenesis. In addition, the plasticity of Th17 cells, which allows a broader dynamics of the Th17/Treg axis in different tumor activities, and the Th17/Th1 axis, which is associated with antitumor mechanisms, are important issues to be taken into account in the immune-oncology field. The modulation of these immune system

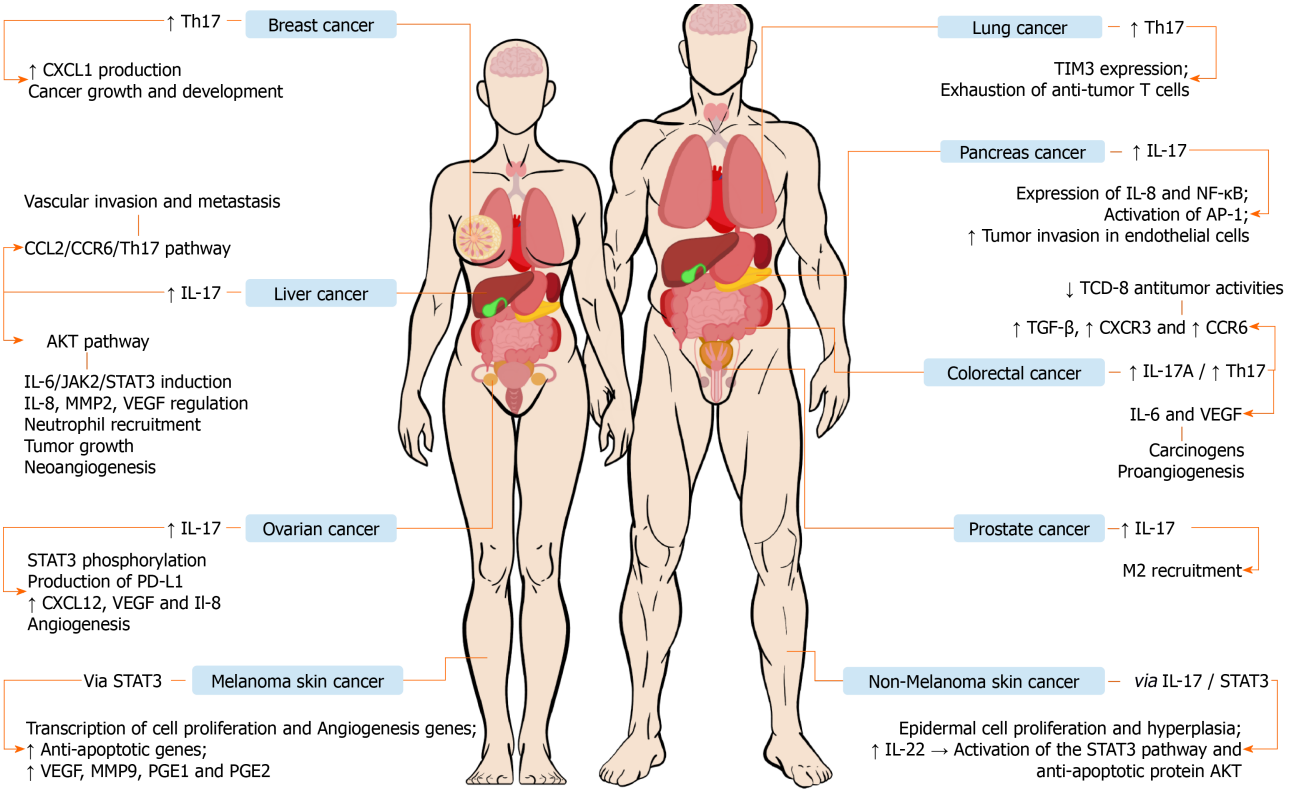


Figure 2 Main protumor mechanisms related to the Th17 immune response profile. STAT3: Signal transducer and activator of transcription 3; MMP2: Matrix metalloproteinases 2; VEGF: Vascular endothelial growth factor; PD-L1: Programmed death-ligand 1; PGE1: Prostaglandins E1; PGE_2: Prostaglandins E2; TIM-3: T-cell immunoglobulin-3; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; AP-1: Activating protein-1.

interplays might be a potential alternative for the development of new therapeutic interventions for various malignancies. Moreover, the role of the IL-27 should be further studied in various types of cancer since an important antitumor effect has been associated with this interleukin in some malignancies. Because IL-17 has shown to be so important in the pathogenesis of several malignant tumors, anti-IL-17 monoclonal antibodies are promising drugs that should be evaluated in numerous neoplasms. Finally, it has to be emphasized that there are several similarities and differences between Th17 responses in various cancers, being it highly dependent on the tumor context. The comprehension of the Th17 immune response in cancer is important not only to predict prognosis, but also to identify new therapeutic possibilities.

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MINIREVIEWS

Human epidermal growth factor receptor 2 targeted therapy in endometrial cancer: Clinical and pathological perspectives

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Abstract

Endometrial cancer is the most common gynecological cancer in developed countries, and its incidence has increased. The majority of patients with endometrial cancer have an early disease and favorable prognosis; however, a significant proportion of endometrial cancer, which mainly comprises high-grade or type II endometrial cancer such as serous, clear cell, and carcinosarcoma, shows advanced/recurrent disease and dismal prognosis. Novel therapeutic development is required for patients with aggressive endometrial cancers. Recent genomic and immunohistochemical analyses revealed human epidermal growth factor receptor 2 (HER2) overexpression/gene amplification in 20%-40% of patients with type II endometrial cancer. Historically, HER2 targeted therapy has been developed for various major cancers, including breast and gastric cancer. Notably, recent advances in HER2 targeted therapy for patients with type II endometrial cancer are also expected to change. Simultaneously, an optimized HER2 test for endometrial cancer as companion diagnostics should be established. In this review, we summarize the recent findings on endometrial cancer, current treatment, optimized HER2 testing, key clinical trials on HER2 targeted therapy, and future directions in aggressive endometrial cancer, including serous carcinoma and carcinosarcoma.

Key Words: Endometrial cancer; Serous carcinoma; Carcinosarcoma; Human epidermal growth factor receptor 2; Chemotherapy; Antibody-drug conjugates

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Core Tip: Endometrial cancer is the most common gynecological cancer in developed countries, and its incidence has increased. A significant proportion of endometrial cancer, which mainly comprises high-grade or type II endometrial cancer, including serous carcinoma and carcinosarcoma, shows a dismal prognosis. Recent molecular analyses revealed human epidermal growth factor receptor 2 (HER2) overexpression/gene amplification in 20%-40% of patients with type II endometrial cancer. Notably, HER2 targeted therapy for type II endometrial cancer has been dramatically developed. We review the recent findings on endometrial cancer, current treatment, optimized HER2 testing, key clinical trials on HER2 targeted therapy, and future directions in these aggressive endometrial cancers.

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INTRODUCTION

Endometrial cancer is the most common gynecologic malignancy, with 65000 cases diagnosed and 12000 deaths in the United States in 2020[1]. In Japan, approximately 16000 cases are diagnosed, 2500 deaths occur annually, and the number of patients is increasing[2,3]. The prognosis is relatively good, with a 5-year survival rate of 80%, and many cases are diagnosed at the localized stage[4]. However, a significant proportion of patients with endometrial cancer still have an advanced or recurrent disease and have a dismal prognosis.

Endometrial cancers can be classified into two types. Type I shows endometrioid morphology, which accounts for 80% of endometrial cancers, and type II shows non-endometrioid morphology, such as serous carcinoma, clear cell carcinoma, and carcinosarcoma, which account for the remaining 20%. Each type of carcinoma is known to have a different background and clinical course. Type I is reportedly caused by unopposed estrogen, and obesity is a trigger for carcinogenesis; it is diagnosed at a young age, has low grade and early stage, and is usually curable by surgery. In contrast to type I, type II is more common in elderly patients, occurs on a background atrophic endometrium, is diagnosed at an advanced stage, and is resistant to chemotherapy, resulting in a poor prognosis[5].

An analysis of The Cancer Genome Atlas revealed different molecular genetic pathways that are present in these two types. The results show that endometrial cancers can be divided into four clusters: *POLE* ultramutated (cluster 1), microsatellite instability hypermutated (cluster 2), copy-number low, endometrioid (cluster 3), and copy-number high; serous-like (cluster 4). Uterine serous carcinoma (USC), classified as Type II, accounts for the majority of cluster 4 cases[6]. Differences in common genomic alterations by cluster were characterized by *PTEN, PIK3R1, FBXW7*, and *KRAS* alterations in cluster 1, *KRAS* alterations in cluster 2, *CTNNB1* alterations in cluster 3, *TP53, FBXW7, PPP2R1A* alterations, *ERBB2*, and *CCND1* amplification in cluster 4[6].

Surgical treatment is appropriate for early-stage endometrial cancer, and chemotherapy and radiation therapy should be considered according to the risk of recurrence after resection. Women with low-risk endometrial cancer undergoing surgical treatment alone and no adjuvant treatment are indicated. For women with high-risk endometrial cancers undergoing surgery, adjuvant chemotherapy with or without radiation should be offered[7,8]. Although there is no consensus on adjuvant treatment among intermediate-risk cancers, some clinicians may offer adjuvant chemotherapy and/or radiation[5]. For advanced or recurrent uterine cancer, carboplatin, paclitaxel, adriamycin, and cisplatin are often chosen as chemotherapy regimens based on the GOG 122L[9], GOG 209L[10], and JGOG 2043 trials[11]. Furthermore, low-grade endometrial cancer with positive estrogen receptor and progesterone receptor can be expected to benefit from hormone therapy[12].

Molecular targeted agents for patients with endometrial cancer have been developed, similar to other solid tumors. In recent years, the efficacy of pembrolizumab has been demonstrated in solid tumors of microsatellite instability-high (MSI-H)/mismatch repair deficient (dMMR)[13,14]. MSI-H/dMMR is found in 20%-30% of endometrial cancers[6,15]. The combination of pembrolizumab and lenvatinib has been shown to be effective in patients with microsatellite stable/mismatch repair proficient, regardless of histologic subtype, with 40% of participants having type II, serous/clear cell carcinoma, and type I[16]. Notably, human epidermal growth factor receptor 2 (HER2) targeted therapy for patients with endometrial cancer has demonstrated practice-changing efficacy in the recent decade. In this review, we aim to overview recent advances in HER2 targeted therapeutics and HER2 testing for endometrial cancer.

HER2 AMPLIFICATION AND OVEREXPRESSION IN ENDOMETRIAL CANCER

HER2 is a cell surface receptor named after human epidermal growth factor receptor (EGFR)-related 2 in the 1980s. It is encoded by the *ERBB2* gene on chromosome 17. It is known to belong to the EGFR family that includes other receptors: EGFR, HER3, and HER4. When *HER2* is amplified, HER2 is overexpressed and forms active dimers that induce the downstream RAS/RAF/MAPK and PI3K/AKT pathways without ligand stimulation. This, in turn, activates the downstream pathway, contributing to oncogenesis[17,18].

HER2 amplification/overexpression is known to occur in a variety of solid tumors, including about 20% in breast cancer[17], 10%-20% in gastric cancer, 2%-10% in colorectal cancer, 5%-20% in biliary tract cancer, 10% in bladder cancer, and 2%-5% in lung cancer[19,20]. In all endometrial cancers, HER2 overexpression and amplification have been reported 18%-80% and 4%-69%[19]. HER2 positivity differs according to histopathological subtype. Although HER2 overexpression and amplification are rarely seen in low-grade endometrioid adenocarcinoma, USC has the highest prevalence of HER2 positivity. The rates of HER2 overexpression and amplification by histologic subtype are shown in Table 1.

HER2-targeted drugs are being used and developed for various malignancies. Currently, trastuzumab, pertuzumab, lapatinib, neratinib, tucatinib[21], trastuzumab emtansine, and trastuzumab deruxtecan[22] have shown clinical activity in HER2-positive breast cancer[19]. For HER2-positive gastric cancer, trastuzumab[23] and trastuzumab deruxtecan[24] have also shown clinical activity. In other carcinomas, HER2-targeted drug therapies are being developed for tumors with HER2 overexpression, amplification, or mutations[25-28]. In endometrial cancer, treatment options are very limited, especially for type II uterine carcinoma, which is resistant to cytotoxic chemotherapy. Therefore, new therapeutic targets and effective new drugs are required.

HER2 overexpression/amplification is currently assessed using immunostaining and fluorescent *in situ* hybridization (FISH)/dual-color *in situ* hybridization. However, there is variability in positive/negative results depending on the type of cancer. In this article, we summarize the methods used to assess HER2 in type II endometrial cancer and the related development of HER2-targeted drugs.

HER2 TESTING IN ENDOMETRIAL CANCER; PATHOLOGICAL PERSPECTIVE

A significant proportion of endometrial cancers show HER2 overexpression or gene amplification[19,29-31]. The percentage of HER2 positive cases varies by histological type, with serous carcinomas having the highest prevalence of HER2 positivity[29-31]. Standardization of HER2 testing accompanied with evidence-based treatment has not been performed for all endometrial cancers; thus, HER2 testing methods are currently optimized for each histological type, specifically in serous carcinoma and carcinosarcoma.

HER2 testing as companion diagnostics has been best established for breast and gastric cancers, and ASCO/CAP guidelines have been provided[32,33]. The HER2 assessment algorithm is based on the detection of protein overexpression by immunohistochemistry (IHC) and gene amplification by ISH to determine HER2-positive/-

Table 1 Human epidermal growth factor receptor 2 overexpression and gene amplification in endometrial cancer

Histological type	HER2 overexpression	*HER2* gene amplification	Ref.
Overall	4%-69%	18%-80%	[19,45,55]
Endometrioid (low grade)	rare	rare	[29,45]
Endometrioid (high grade)	8%-31%	15%-29%	[29,45]
Serous carcinoma	20%-60%	13%-29%	[29,42,43,45]
Clear cell carcinoma	33%-70%	22%-50%	[29,55]
Carcinosarcoma	0-25%	13%-20%	[38,48-51]

negative cases[32,33]. Although HER2 overexpression has been reported in other types of carcinomas, in most cases, these two HER2 testing methods are used[19,34] or modified (*e.g.*, in colorectal cancer[35,36]).

In endometrial cancer, HER2 testing has become more clinically relevant in serous carcinoma[37] and carcinosarcoma[38,39], with the accumulating knowledge of HER2 testing accompanied by ongoing therapeutic developments.

HER2 TESTING IN SEROUS CARCINOMA

Serous carcinoma accounts for up to 10% of endometrial cancers; however, it is aggressive and responsible for almost 40% of endometrial cancer deaths[40]. Histopathologically, serous carcinoma is usually composed of tumor cells with marked nuclear pleomorphism and presents a complex papillary pattern of typically small, bud-like papillae, irregular slit-like glands, an endometrioid-like glandular pattern, solid nests and sheets, and microcysts[41]. HER2 is overexpressed in 20%-60% of serous carcinomas[29,42-45]; however, less frequent HER2 gene amplification has been reported[43,44]. Notably, the staining pattern of HER2 in serous carcinoma cells has been well studied; approximately 75% of HER2 expressing carcinoma cells reportedly show a basolateral/Lateral membranous staining pattern, similar to that of gastric adenocarcinoma[37]. HER2 assessment of serous carcinoma has been performed using various methods, mainly based on the breast cancer criteria[42-44]. However, an optimal HER2 testing method specific for endometrial serous carcinoma has not been established in the clinical trial setting.

Recently, a randomized phase 2 clinical trial of carboplatin/paclitaxel *vs* carboplatin/ paclitaxel/trastuzumab in advanced or recurrent HER2 positive serous carcinoma demonstrated the survival benefit of adding trastuzumab[46]. Both prolonged progression-free survival (PFS) and overall survival (OS) were observed in the trastuzumab arm. For patient enrollment in this clinical trial, HER2 status was determined based on the modified 2007 ASCO/CAP breast criteria (Figure 1)[37]. Serous carcinoma showing intense complete or lateral/basolateral membranous HER2 staining in more than 30% of tumor cells were classified as score 3+, and score 2+ was assigned when intense complete or lateral/basolateral membrane staining was seen in ≤ 30%, or weak to moderate staining in ≥ 10% of tumor cells. FISH was performed only in tumors with an IHC score of 2+, and a HER2/CEP17 ratio of ≥ 2.0 was considered as amplified. Although this HER2 assessment criterion was justified in the context of the treatment effect of trastuzumab-containing regimens, it is still uncertain whether this HER2 testing algorithm should be used in patients receiving HER2 targeted drugs with a different mode of action from that of trastuzumab. For example, HER2-antibody drug conjugate (ADC) has been shown to be clinically effective in patients with low-level HER2 expressing breast cancer who have been assessed as HER2-negative according to the ASCO/CAP breast cancer criteria established for the prediction of response to trastuzumab[47]. The HER2 testing algorithm was optimized to maximize patient benefit and safety for each HER2 targeted therapy.

HER2 TESTING IN UTERINE CARCINOSARCOMA

Uterine carcinosarcoma (UCS) is a rare and high-grade subtype of endometrial cancer (WHO2020) and is characterized by the presence of both carcinomatous and

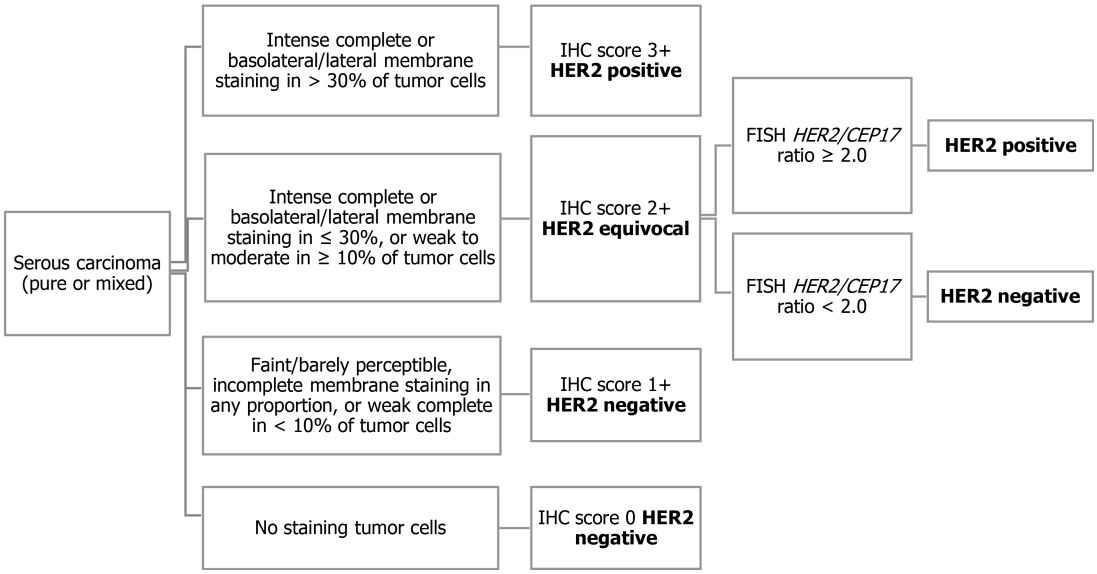


Figure 1 Human epidermal growth factor receptor 2 testing for serous carcinoma used in the Phase 2 clinical trial[37,46]. IHC: Immunohistochemistry; FISH: Fluorescent in situ hybridization.

sarcomatous components that are usually intimately admixed. The carcinomatous component is usually a high-grade endometrioid, serous, clear, or nonspecific carcinoma. HER2 is a promising therapeutic target for UCS. *HER2* gene amplification has been reported in 13%-20% of UCS cases[48,49]. Additionally, HER2 overexpression (IHC score 3+) reportedly ranges from 0 to 25%[38,49-51].

An evidence-based HER2 testing protocol for UCS has not been fully established; thus, several researchers have adopted HER2 testing protocols and assessment methods based on ASCO/CAP recommendations for HER2 testing for breast cancer and gastroesophageal cancer.

Recently, we proposed several requirements for HER2 testing in UCSs[39]. Our evaluation method has been used in the ongoing phase 2 clinical trial of HER2 ADC in patients with recurrent/metastatic UCS (STATICE trial)[52]. In this study, we identified that most UCS showed lateral/basolateral staining patterns (Figure 2), similar to endometrial serous carcinoma and gastric adenocarcinoma[39]. Based on our previous observations, we concluded that a HER2 testing protocol for UCS should contain the following requirements[39]: (1) Established pre-analytical factors of HER2 IHC[32,33] should be carefully controlled; (2) One representative section containing carcinoma components should be submitted from the hysterectomy specimen; (3) HER2 IHC should be performed using an IVD kit or a laboratory-developed test with appropriate quality control[32,33]; (4) Positive lateral/basolateral membranous staining patterns should be considered as positive, according to the 2016 ASCO/CAP gastric cancer criteria[32]; and (5) The proportion of HER2 expressing tumor cells should be determined as an approximate number of HER2-positive tumor cells divided by that of total tumor cells (both carcinoma and sarcomatous elements). Unfortunately, we did not provide supporting data for the best scoring system based on the patient clinical outcomes. However, we would report the correlation between treatment efficacy and HER2 score in patients with UCS in 1-2 years.

FUTURE DIRECTION OF HER2 TESTING IN ENDOMETRIAL CANCER

Most quality assurance of HER2 testing should be performed in accordance with well-established methods for breast and gastric cancer. In addition, we should consider specific issues for HER2 testing in endometrial cancers, including correlation with specific treatment response, intratumoral heterogeneity of HER2 status, discordant HER2 status between the primary site and metastasis, improvement of interobserver reproducibility of HER2 assessment, and use of liquid biopsy in the future.

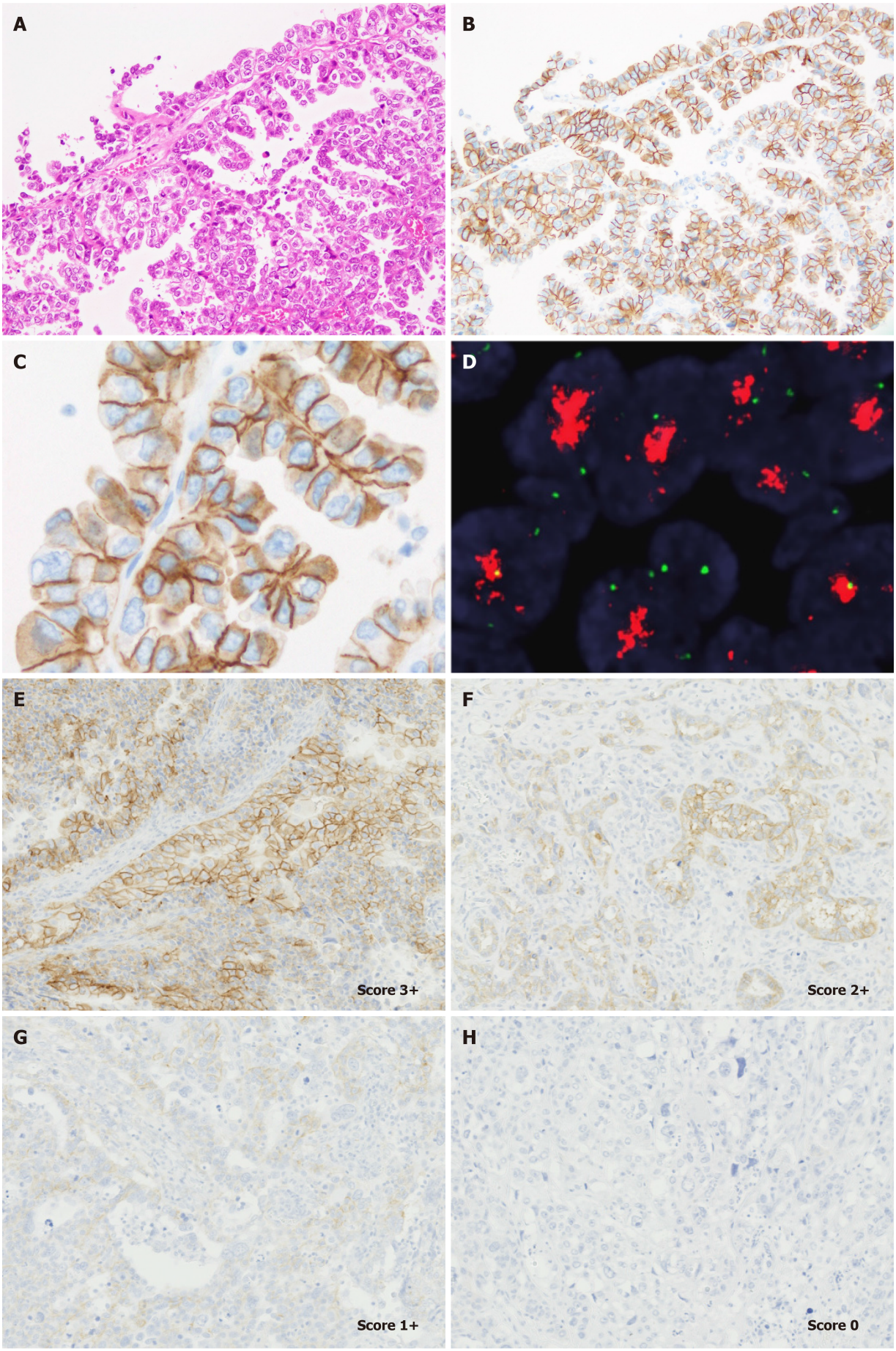


Figure 2 Representative staining patterns of human epidermal growth factor receptor 2 and fluorescent in situ hybridization results in

uterine carcinosarcoma. Serous-like carcinoma component (A) shows diffuse strong membranous staining of Human epidermal growth factor receptor 2 (HER2) (B). Most tumor cells show lateral or basolateral membranous staining patterns (C). *HER2* amplification observed in these tumor cells using fluorescent in situ hybridization (D). Representative images of HER2 score according to ASCO/CAP gastric cancer criteria. Score 3+ (E), score 2+ (F), score 1+ (G), and score 0 (H) (× 200). A (H&E, × 100); B, E-G, and H (HER2, × 100); C (HER2, × 400). FISH: Fluorescent in situ hybridization; H&E: Hematoxylin and eosin.

CURRENT THERAPIES AND FUTURE DIRECTIONS FOR HER2 TARGETED THERAPIES FOR UTERINE SEROUS CARCINOMA AND UTERINE CARCINOSARCOMA

Trastuzumab is the most popular HER2-targeted drug. Several case reports have shown that trastuzumab had clinical activity in patients with endometrial cancer[53, 54]. Fleming *et al*[55] investigated the efficacy of trastuzumab as a single agent in HER2-positive endometrial carcinoma in a GOG study (Table 2). However, the overall response rate was 0%, and no significant clinical activity was observed. A possible issue was that many endometrioid carcinomas with low HER2 positivity were screened, and the study had early termination with poor accrual. Lapatinib is a dual inhibitor of EGFR and HER2 used in HER2-positive breast cancer. The GOG 229D[56] trial examined the efficacy of lapatinib in uterine cancer (n = 2 for HER2-positive) in a phase II trial. The response rate was 3%, the median PFS was 1.82 mo, and clinical activity could not be demonstrated. This lack of clinical activity could be explained by the unselected patient population. Therefore, further studies are warranted to highlight type II endometrial carcinoma patients with high HER2-positivity.

Fader *et al*[46] conducted a randomized phase II trial to evaluate the effect of trastuzumab on carboplatin and paclitaxel, the standard of care for HER2-positive USC. The median PFS [12.6 mo *vs* 8.0 mo; hazard ratio (HR) = 0.44, 90% confidence interval (CI): 0.26-0.76, P = 0.005] was significantly different between the two groups. Updated analysis (median PFS 12.9 mo *vs* 8.0 mo; HR = 0.46, 90%CI: 0.28-0.76; P = 0.005, median OS 29.6 mo *vs* 24.4 mo; HR = 0.58, 90%CI: 0.34-0.99; P = 0.46) showed clinically significant benefit[57]. The addition of trastuzumab to carboplatin and paclitaxel chemotherapy represents a new standard treatment for USC. In the NCCN guidelines, carboplatin, paclitaxel, and trastuzumab combination therapy are recommended in Category 2A for advanced or recurrent USC[58].

Several basket trials in HER2-positive solid tumors, including endometrial carcinoma, have been reported. MyPathway is a phase II, multiple basket study with patients with advanced refractory solid tumors harboring molecular alterations such as *HER2, BRAF, EGFR*, and the Hedgehog pathway[25]. Patients with *HER2* alterations were treated with pertuzumab plus trastuzumab. Eight patients with HER2-positive endometrial carcinoma received trastuzumab plus pertuzumab. No responses were observed. The efficacy of trastuzumab emtansine has also been evaluated in a phase II basket trial, including HER2-positive endometrial carcinoma[59]. This trial demonstrated that 18 patients with uterine cancer (subtype unknown) were included, and two patients had a complete response and two had a partial response (PR), suggesting high efficacy.

Currently, several clinical trials of HER2-targeted drugs are ongoing. The DESTINY-PanTumor02 trial is a basket trial to evaluate the efficacy of trastuzumab deruxtecan in HER2-positive tumors, including endometrial carcinoma (NCT04482309). A phase I trial evaluating combination therapy with trastuzumab deruxtecan and olaparib in HER2-positive USC is also ongoing (NCT04585958). In HER2-positive UCS, the STATICE trial is a phase II trial to evaluate the efficacy of trastuzumab deruxtecan (UMIN00002956, NCCH1615)[52].

Novel therapeutic agents targeting HER2 have emerged. Trastuzumab duocarmazine (known as SYD985) is a novel HER2-targeted ADC that combines trastuzumab with duocarmazine, a DNA alkylating agent, as a payload. Trastuzumab duocarmazine has shown preclinical anti-tumor activity in USC[60]. Banerji *et al*[61] demonstrated phase 1 dose-escalation and dose-expansion study in breast, gastric, urothelial, and endometrial carcinomas that express HER2. Thirteen patients with endometrial cancer were included in the dose-expansion cohort. Five patients (39%) had PR, and the median PFS was 4.3 mo[61]. Treatment-related serious adverse events were reported in 11% of patients, and the frequency of cardiac toxicity did not increase compared with previous anti-HER2 drugs. Most patients had ocular adverse events, such as conjunctivitis, dry eye, and lacrimation. Trastuzumab duocarmazine has a manageable safety toxicity profile[61]. Further investigation of trastuzumab duocarmazine is ongoing in phase III trials for HER2-positive breast cancer (TULIP study,

Table 2 Human epidermal growth factor receptor 2-targeted therapies for uterine serous carcinoma and carcinosarcoma

Trial	Phase	Participants	Treatment	Efficacy/indentifer
GOG181B[55]	II	HER2-positive EC (n = 33, endometrioid: 13, serous: 11, clear: 3, others: 6)	Trastuzumab	ORR 0%, mPFS 1.84 mo, mOS 7.8 mo
GOG229D[56]	II	Persistent or recurrent EC (n = 31, endometrioid: 16, serous: 7, clear: 3, others: 5, HER2-positive: n = 2)	Lapatinib	mPFS 1.82 mo, mOS 7.33 mo
Fader *et al*[46,57]	II	HER2-positive USC (n = 61)	carboplatin+paclitaxel+trastuzumab *vs* carboplatin+paclitaxel	mPFS 12.9 mo *vs* 8.0 mo, mOS 29.6 *vs* 24.4 mo
MyPathway[25]	Phase IIa Multiple basket study	Solid tumor(HER2-positive EC, n = 7)	Trastuzumab+pertuzumab	ORR 0%(EC)
Li *et al*[59]	II	HER2 amplified cancer (endometrial cancer: n = 18)	Trastuzumab emtansine	Endometrial cancers: CR 2, PR 2
DESTINY-PanTumor02[77]	II	HER2 expressing tumor (urothelial, biliary tract, cervical, endometrial, ovarian, pancreatic, rare tumors)	Trastumab deruxtecan	NCT04482309
Veneris[78]	I	HER2-positive USC	Trastuzumab deruxtecan+olaparib	NCT04585958
Banerji *et al*[61]	I	Dose-expansion cohort: HER2-positive breast, gastric, urothelial, endometrial cancer	SYD-985 (Trastuzumab duocarmazine)	Endometrial cancer: ORR 39%, mPFS 4.3 mo
Koper[79]	I	HER2-positive solid tumor	SYD-985 (Trastuzumab duocarmazine)+Niraparib	NCT04235101
Hendriks[80]	II	HER2-positive endometrial cancer	SYD-985 (Trastuzumab duocarmazine)	NCT04205630
STATICE[52]	II	HER2-positive UCS	Trastuzumab deruxtecan	UMIN00002956 (NCCH1615)
Makker[81]	II	HER2-positive endometrial cancer, UCS	ZW25	NCT04513665
Ramos[82]	II	HER2-positive solid tumors	Tucatinib and trastuzumab	NCT04579380

EC: Endometrial cancer; USC: Uterine serous carcinoma; UCS: Uterine carcinosarcoma; CR: Complete response; mOS: Median overall survival; mPFS: Median progression-free survival; ORR: Objective response rate; PR: Partial response.

NCT03262935), phase II trials for HER2-positive endometrial cancer (NCT04205630), and phase I trial for HER2-positive solid tumor combination with niraparib (NCT04235101).

There are important considerations for future studies. Anti-HER2 therapy has been successful in HER2-positive breast cancer, but most of these drugs have not been successful in non-breast HER2-positive solid tumors. One explanation for these differences is the pattern of HER2 expression and heterogeneity within the tumor. Since the HER2 expression pattern of USC and UCS is similar to that of gastric cancer, but not breast cancer, it is possible that the same HER2-targeted therapy may have similar effects.

The first consideration may be to focus on combination therapies and ADC drugs rather than HER2 blockade alone in endometrial cancer. The second consideration is to overcome the resistance to HER2 targeted agents. Mechanisms of resistance to HER2 targeted therapy have been studied and can be classified into four groups: intratumoral heterogeneity, alterations in the binding site, activation of downstream signals, and overexpression of other HER2 family members[62]. The strategy against intratumoral heterogeneity could be a bystander effect of clinical activity against not only targeted cells but also surrounding cells. A novel HER2 ADC drug, trastuzumab deruxtecan, and trastuzumab duocarmazine showed a bystander effect *in vitro* and *in vivo*, and showed anti-tumor activity in clinical trials with low HER2 positivity[47,60,63-65]. Alteration in the binding site of HER2 and extracellular domain shedding could be overcome using irreversible inhibitors. In preclinical studies, afatinib and neratinib are irreversible pan-HER inhibitors that show anti-tumor activity in USC and gynecologic carcinosarcoma[66-68]. Tucatinib is an HER2 and HER3 kinase inhibitor, combination with trastuzumab are investigated in baskets study of Solid tumor with HER2 alterations, including uterine neoplasms. In downstream pathway alterations, the gain of function in PI3K is well known as resistance to HER2. Combined HER2 and PIK3CA dual inhibition using neratinib and taselisib were effective in cell lines and

xenograft models of USC[69,70]. Of overexpression of other HER2 family members, HER3 overexpression plays an important role. The formation of HER2-HER3 heterodimers is most related to resistance to anti-HER2 therapy. Pertuzumab is an HER2 antibody that binds to a different epitope from that of trastuzumab and inhibits dimerization. In breast cancer, the combination of trastuzumab and pertuzumab improved clinical outcomes[71]. Pertuzumab and trastuzumab showed anti-tumor activity in USC cell lines[72]. Although these drug combinations showed no clinical benefit in the Mypathway trial with HER2 amplification in endometrial cancer[25], further investigation is warranted. Several HER2 bispecific antibodies have been developed that simultaneously bind to two distinct HER2 epitopes, the same domain as trastuzumab and pertuzumab[73]. Researchers demonstrated a phase 1 basket trial to evaluate ZW25, one of HER2 bispecific antibodies, in HER2 positive solid tumors, including endometrial cancer. Of 17 evaluable patients, seven patients (41%) had an objective response, and the median PFS was 6.2 mo[74]. ZW25 is investigated in phase 2 clinical trial of HER2 overexpressed advanced endometrial cancer and carcinosarcomas.

In addition, there is rapidly growing HER2-directed immunotherapy in patients with HER2-positive solid tumors[19,75]. Several drugs with a different target, such as bispecific antibodies, immune-stimulating conjugates, vaccines, and adoptive T-cell therapies, are under investigation[76]. The bispecific HER2/CD3 antibodies BTRC4017A, GBR-1302 and M802 induce cytotoxic effect by interaction with HER2 on tumor cell and CD3 on cytotoxic T cell. NJH395 are immune-stimulating antibody conjugates which HER2 antibody links to payload as toll-like receptor 7 (TLR7) and TLR8. Stimulating TLR activated natural killer cells and antigen-presenting cells and facilitate invasion of CTLs to tumor tissues. PRS-343 increases tumor lymphocyte invasion *via* targeting HER2 and CD137 (4-1BB). CD137 is known as a co-stimulating factor of T cell activation[73]. We expect a further investigation of these drugs in patients with endometrial cancer.

CONCLUSION

In this review, we provided an overview of HER2-overexpression/amplification in endometrial cancer, pathological evaluation methods, and the current status of HER2-targeted therapies. With the advent of precision medicine, the development of therapies targeting biomarkers has become increasingly advanced. In the development of anti-HER2 inhibitors and ADC drugs targeting HER2, it may be important to develop not only a single drug but also combination therapies. Since there are limited therapeutic agents for endometrial cancer, especially for type II, the development of HER2-targeted therapy is urgently needed.

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MINIREVIEWS

Role of lenalidomide in the treatment of peripheral T-cell non-Hodgkin lymphomas

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Abstract

T-cell lymphomas (TCLs) represent a group of lymphoid neoplasms characterized by an aggressive clinical course, even after an anthracycline-containing regimen. Novel agents for patients with relapsed/refractory TCL are urgently needed. Lenalidomide is an oral drug with immunomodulatory, antiangiogenic and direct antineoplastic effects. These peculiar mechanisms of action make TCL an attractive target for lenalidomide. We have identified five clinical trials in which lenalidomide monotherapy was investigated to treat TCL, including cutaneous TCL (CTCL) and adult T-cell lymphoma/leukemia (ATLL). In the ATLL-002 study, the overall response rate (ORR) was 42% and median progression-free survival (PFS) and overall survival were 3.8 mo and 20.3 mo, respectively. In a phase II trial for CTCL, ORR was 28% and median PFS and overall survival were 8 mo and 43 mo, respectively. For nodal peripheral TCL, ORR was between 10% and 43% in three clinical trials, with a median PFS of about 4 mo, even if some patients had a durable response. Overall toxicity is manageable and grade 3-4 events are mainly hematological and reversible. Combination strategies did not improve PFS. In conclusion, lenalidomide could represent a suitable treatment option for relapsed/refractory TCL, especially for neoplasms with a T-follicular helper origin, such as angioimmunoblastic TCL.

Key Words: T-cell lymphomas; Lenalidomide; Therapy; Survival; Safety; T follicular helper

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Core Tip: T cell lymphoma (TCL) are rare. Lenalidomide is an oral drug with an immunomodulatory, antiangiogenic and direct antineoplastic effect. These peculiar mechanisms of action makes TCL an attractive target for lenalidomide. We have

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identified 5 clinical trials in which lenalidomide monotherapy was investigated to treat TCL, including cutaneous TCL and adult T-cell lymphoma/leukemia. Overall response rate was between 10 % and 43%, with prolonged response in a significant proportion of cases and manageable toxicity. Lenalidomide could represent a suitable treatment option for R/R TCL, especially for neoplasms with a T-follicular helper origin, such as angioimmunoblastic TCL.

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INTRODUCTION

Peripheral T-cell lymphomas (TCLs), including primary nodal, extranodal, leukemic and cutaneous TCL (CTCL), are a heterogeneous group of lymphoid neoplasms characterized by aggressive clinical course and poor prognosis, representing about 10%-20% of non-Hodgkin lymphoma cases[1-3]. Due to its rarity, the treatment approach has historically been similar to that used for aggressive B-cell neoplasms, such as diffuse large B-cell lymphoma (DLBCL)[3,4]. However, except for anaplastic lymphoma kinase (ALK)-positive anaplastic lymphoma (ALCL), treatment efficacy is limited compared to DLBCL, disease relapse is frequent even after high-dose therapy and autologous stem-cell transplantation, and long-term responses are seldom observed[1,5-8]. Allogeneic stem-cell transplantation often remains the only curative option, but many elderly and/or relapsed/refractory (R/R) patients are not eligible, and treatment morbidity and mortality are not negligible[9]. Novel agents approved by the Food and Drug Administration and/or the European Medicines Agency for R/R TCL, as shown in Table 1, include pralatrexate, romidepsin, belinostat, brentuximab vedotin (only for ALCL and CD30-positive CTCL) and mogamulizumab [only for mycosis fungoides and Sézary syndrome (SS)][10-14]. Additional new drugs with different mechanisms of action are listed in the National Comprehensive Cancer Network guidelines, including bortezomib, alemtuzumab, crizotinib, cyclosporine, nivolumab and lenalidomide[15]. Interestingly, several drugs work very well on a few TCL subtypes, while their efficacy is limited for the others; this allows for speculation that disease biology could influence the therapeutic response[16].

Lenalidomide is an immunomodulatory drug initially approved as a treatment for patients with R/R multiple myeloma (MM)[17]. It was successfully used to treat myelodysplastic syndromes, chronic lymphocytic leukemia and R/R B-cell non-Hodgkin lymphoma, including DLBCL, mantle-cell lymphoma and follicular lymphoma[18-22]. Due to its peculiar mechanisms of action, TCL could represent an attractive target for lenalidomide; however, available data are limited[4,14]. According to this background, we would like to briefly summarize the pharmacological properties and to review the clinical efficacy and safety of lenalidomide monotherapy in previously treated TCL.

SEARCH CRITERIA FOR LITERATURE REVIEW

We performed a computerized search in MEDLINE to find full-text publications, in English, published to 2020, which focused on lenalidomide and TCL. We included nodal and extranodal peripheral TCL (PTCL), adult T-cell leukemia-lymphoma (ATLL) and CTCL. The key terms were "T-cell lymphoma OR TCL OR T-cell non-Hodgkin lymphoma OR mycosis fungoides (MF) OR Sézary syndrome (SS) OR cutaneous TCL OR peripheral TCL OR adult T-cell leukemia-lymphoma AND lenalidomide OR treatment OR immunotherapy OR combined modalities". We included prospective clinical trials, retrospective studies, letters to the editor and case reports. For each study, we extracted the following data, when available: number of patients, study design, patient population, treatment regimen, overall response rate (ORR), complete response (CR) rate, time to response (referred to as TTR), toxicity, and survival end-points, such as OS, PFS and duration of response (DOR). OS represents

Table 1 Clinical results of approved novel agents as monotherapy for relapsed/refractory T-cell lymphomas

Ref.	Patients, *n*	Study design	Treatment	Mechanism of action	Histology, (number of patients)	ORR	CR rate	Median PFS	Median DOR	Median OS
O'Connor *et al*[10], 2011	111	Multicenter phase II	Pralatrexate	Antifolate	Total evaluable (109)	29%	11%	3.5 mo	10.1 mo	14.5 mo
					PTCL-NOS (59)					
					ALCL (17)	32%	NA			
					AITL (13)	35%	NA			
					MF (12)	8%	NA			
					Other (8)	25%	NA			
						38%	NA			
Coiffier *et al*[11], 2014	130	Multicenter phase II	Romidepsin	Histone deacetylase inhibitor	Total evaluable -130	25%	15%	4 mo	28 mo	11.3 mo
					PTCL-NOS (69)	29%	14%			
					ALCL ALK- (21)	24%	19%			
					AITL (27)	30%	19%			
					Other (13)	/	/			
O'Connor *et al*[12], 2015	129	Multicenter phase II	Belinostat	Histone deacetylase inhibitor	Total evaluable -120	25.80%	10.80%	1.6 mo	13.6 mo	7.9 mo
					PTCL-NOS (77)	23%	NA			
					ALCL ALK- (13)	15%	NA			
					ALCL ALK+ (2)	/	NA			
					AITL (22)	46%	NA			
					Other (6)	16.60%	NA			
Pro *et al* [13], 2017	58	Multicenter phase II	Brentuximab vedotin	Monoclonal antibody anti-CD30	ALCL	86%	57%	20 mo	25.6 mo	NR (estimated 5-yr OS 60%)
Kim *et al* [14], 2018	186	Multicenter, randomized, phase III	Mogamulizumab	Monoclonal antibody anti C-C chemokine receptor 4	MF or Sézary syndrome	28%	2.70%	7.7 mo	14.1 mo	NR

AITL: Angioimmunoblastic T-cell lymphoma; ALCL: Anaplastic T-cell lymphoma; ALK: Anaplastic lymphoma kinase; CR: Complete response; DOR: Duration of response; MF: Mycosis fungoides; NA: Not available; NR: Not reached; ORR: Overall response rate; OS: Overall survival; PFS: Progression-free survival; PTCL-NOS: Peripheral T-cell lymphomas: not otherwise specified.

the most relevant outcome; PFS could be more accurate but the PFS definition has not been the same in different studies over the years. We included abstracts extracted from the last meetings of the European Hematology Association, American Society of Hematology, and International Conference on Malignant Lymphoma.

MECHANISMS OF ACTION AND PHARMACOLOGICAL CHARACTERISTICS OF LENALIDOMIDE

Lenalidomide is administered orally and rapidly adsorbed, without marked accumulation, even after multiple courses[23]. The drug is mainly excreted unchanged by renal elimination, thus a dose reduction is recommended in patients with reduced creatinine clearance due to renal impairment. Lenalidomide is characterized by an acceptable safety profile, and clinically relevant interactions with other drugs are unlikely[23].

The multiple mechanisms of action, as shown in Figure 1, include an immunomodulatory, antiangiogenic and direct antineoplastic effect[24]. Lenalidomide may be able to restore the function of the T-cell immune synapse and to suppress regulatory T-cells (*i.e.*, Tregs)[25,26]. Moreover, it could activate CD8-positive T-cells and favor a shift of T-helper (*i.e.*, T_H) response towards a T_H1 *vs* a T_{H2} subtype[27,28]. Moreover, it could improve natural killer (NK)-mediated immune function and NK cell activation, leading to the induction of a primarily NK-mediated tumor cells apoptosis[28,29]. Moreover, lenalidomide could inhibit the production of cytokines with a pro-inflammatory activity, such as interleukin (IL)-1, IL-6, IL-12 and tumor necrosis factor-α, while the production of anti-inflammatory molecules, such as IL-10, is increased[30].

The antiangiogenic effect is achieved by blocking the migration and adhesion of endothelial cells and by inhibiting the formation of microvessels (reduced microvessels density)[28]. In mantle-cell lymphoma mouse models, the drug could cause a depletion of immune cells associated with lymphomagenesis, such as monocytes and macrophages[31]. Moreover, a direct inhibitory effect of lenalidomide on the vascular endothelial growth factor production was demonstrated and was associated with an increased SPARC expression[32,33].

The antiproliferative effect is carried out by directly inducing G1 growth arrest of the cell cycle and apoptosis[34]. A relevant discovery was represented by the identification of cereblon, a component of a cullin-RING E3 ubiquitin ligase enzyme complex, which includes the deoxyribonucleic acid damage binding protein 1, cullin 4 and the cullin regulator 1[35,36]. After the direct binding of lenalidomide, cereblon leads to the ubiquitination and subsequent degradation of the substrate proteins Aiolos (IKZF3) and Ikaros (IKZF1), which are lymphoid transcription factors, with consequent cytotoxic and immunomodulatory effects[37,38]. In DLBCL, especially nongerminal center B-cell-like DLBCL, a direct, cereblon-dependent capability of lenalidomide to kill neoplastic cells was observed, through deregulation of interferon regulatory factor 4[39]. Remarkably, the antiproliferative effect can occur in a p53-independent manner[40].

LENALIDOMIDE IN THE TREATMENT OF TCLS

ATLL

ATLL represents an uncommon neoplasm linked to the human T-lymphotropic virus type 1 infection and characterized by an aggressive course with poor prognosis[41]. Although rare in Western countries, it represents a common TCL in Japan, where human T-lymphotropic virus type 1 is endemic[41]. ATLL is subdivided into four subtypes: smoldering; chronic (often with an indolent course); lymphoma; and acute (with a very aggressive behavior)[2,41]. Anthracyclines-containing regimens, such as cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP), showed limited efficacy, and most patients are refractory or relapse after an initial response[42]. R/R ATLL, due to the rarity of disease, has been frequently excluded from clinical trials investigating novel agents[42]. Ogura *et al*[43] investigated lenalidomide in relapsed ATLL or PTCL in a phase I study, in which 14 patients were enrolled. Maximum tolerated dose was 25 mg daily given continuously; out of 9 ATLL cases, 3/9 achieved a partial response (PR), leading to the design of a phase II study (ATLL-002), in which 26 relapsed or recurrent Japanese ATLL cases were enrolled[43,44]. The patients had received a median of two previous regimens (range: 1-4). Interestingly, 2 patients received lenalidomide for more than 1 year. As represented in Table 2, ORR was 42% (11/26 cases), with 4 CR and 1 unconfirmed CR; out of 11 responders, 2 were previously treated with mogamulizumab[44]. Median TTR, median PFS and OS were 1.9 mo, 3.8 mo and 20.3 mo, respectively, with manageable toxicity. According to disease subtype, ORR was 33% for acute ATLL and 57% for lymphoma ATLL[44]. Interestingly, in another Japanese experience, 2/4 relapsed ATLL cases after allo-stem-cell

Table 2 Clinical efficacy of lenalidomide single-agent in T-cell lymphomas

Ref.	Patient, *n*	Study design	Treatment	Histology (number of patients)	ORR	CR rate	Median PFS	Median DOR	Median OS
Ishida *et al*[44], 2016	26	Multicenter phase II	25 mg continuously until progression or unacceptable toxicity	ATLL (26)	42%	19%	3.8 mo	NR	20.3 mo
Querfeld *et al* [52], 2014	32	Multicenter phase II	25 mg for 21 d of a 28-d cycle; initial dose was reduced to 10 mg, with the possibility of increasing by 5 mg every cycle, until a maximum of 25 mg, until progression or up to 2 yr for SD or PR, while patients in CR received two additional cycles	Total evaluable (29)	28%	/	8 mo	10 mo	43 mo
				Mycosis fungoides (19)	36.8%				
				Sézary syndrome (13)	15.4%				
Zinzani *et al* [56], 2011	10	Phase II, bi-centric	25 mg for 21 d of a 28-d cycle for 4 cycles as induction phase; After the 4th cycle, patients who achieved at least a SD continued for other eight cycles as maintenance	PTCL-NOS (10)	30%	30%	NA	13 mo	NA
Morschhauser *et al*[51], 2013	54	Multicenter phase II	25 mg on d 1-21 of a 28-d cycle, until progression or unacceptable toxicity, for a maximum of 2 yr	Total evaluable (54)	22%	11%	2.5 mo (4.6 mo in AITL)	3.6 mo	NA
				AITL (26)	31%	15%			
				PTCL-NOS (20)	20%	NA			
				CTCL (3)	NA	NA			
				ALCL (3)	NA	NA			
				Cutaneous ALCL (1)	NA	NA			
				Extranodal NK/T-cell, nasal type (1)	NA	NA			
Toumishey *et al* [57], 2015	39	Multicenter phase II	25 mg daily on d 1-21 of a 28-d cycle, until progression or unacceptable toxicity	Total evaluable (39)	26%	7.7%	4 mo	13 mo	12 mo
				ALCL (10)	10%	/			
				AITL (9)	33%	11.1%			
				PTCL-NOS (14)	43%	14.3%			
				Other (6)	/	/			

AITL: Angioimmunoblastic T-cell lymphoma; ALCL: Anaplastic T-cell lymphoma; ATLL: Adult T-cell lymphoma/leukemia; CTCL: Cutaneous T-cell lymphoma; CR: Complete response; DOR: Duration of response; NA: Not available; NK: Natural killer; NR: Not reached; OS: Overall survival; PR: Partial response; ORR: Overall response rate; PFS: Progression-free survival; PTCL-NOS: Peripheral T-cell lymphomas: Not otherwise specified; SD: Stable disease.

transplantation achieved a CR, and 1 of them showed a prolonged remission[45].

Even if these findings have not been confirmed outside Japan, lenalidomide could represent a promising option for R/R ATLL, as single-agent or in the context of combination strategies[46].

CTCLS

CTCLs are a heterogeneous group of TCL, and cell of origin is represented by skin-homing, mature, CD4-positive T_H lymphocytes[47]. MF and SS are the most common subtypes and frequently relapse after systemic therapy; R/R MF/SS are not curable and characterized by dismal prognosis[48]. Moreover, treatment toxicity is elevated after cytotoxic therapy, thus biological agents are needed to improve therapeutic efficacy with limited toxicity[47,48]. As there is a prevalent T_H2 response with a reduction of CD8-positive T-cells in MF/SS, especially in advanced-stage disease, the immunomodulatory characteristics of lenalidomide represent a strong rationale for its use in CTCL patients[49,50].

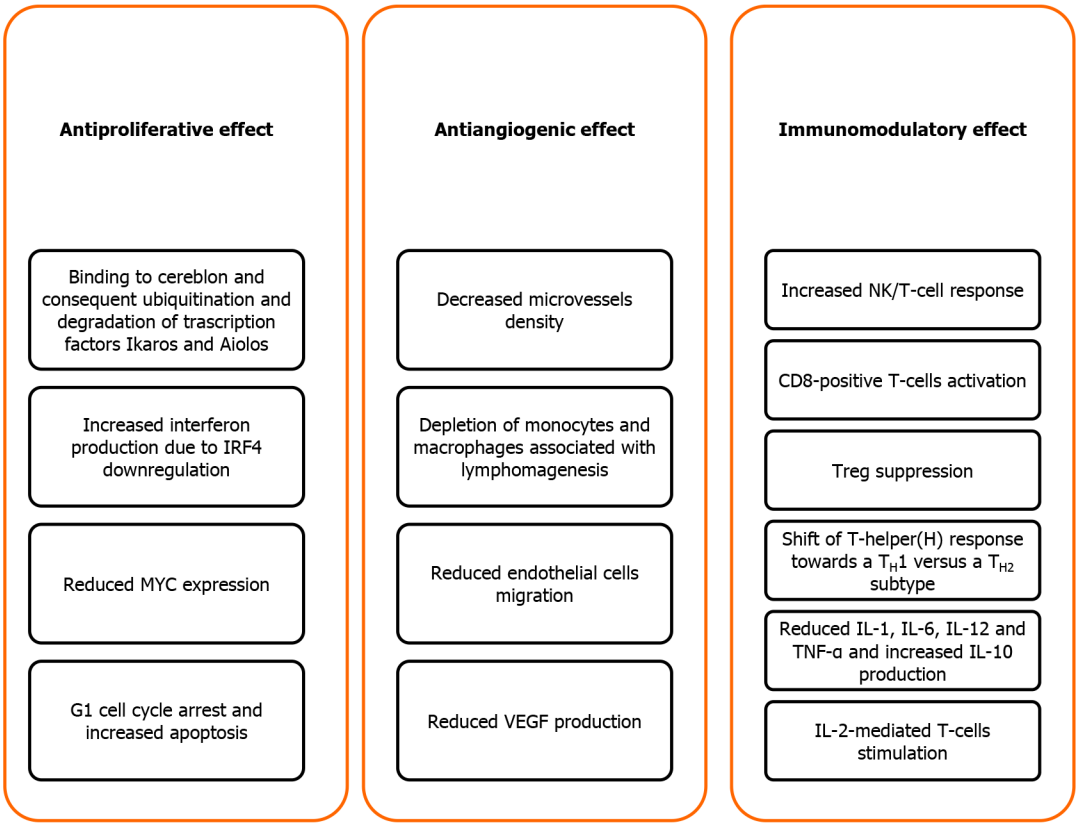


Figure 1 Lenalidomide mechanisms of action. IL: Interleukin; VEGF: Vascular endothelial growth factor.

Three MF patients were included in the phase II, single-arm EXPECT trial, in which lenalidomide dose was 25 mg once daily on days 1-21 of a 28-d cycle, given until progressive disease (PD) or unacceptable toxicity, for a maximum of 2 years. Unfortunately, treatment response for MF patients was not reported[51]. As illustrated in Table 2, Querfeld *et al*[52] enrolled in a phase II trial 32 patients with stage IB to IVB MF (18 cases), erythrodemic MF (3 cases) and SS (11 cases), who failed at least one prior therapy (skin-directed or systemic therapy). Lenalidomide was administered at a daily dose of 25 mg for 21 d of a 28-d cycle to the first 19 patients; the protocol was amended due to fatigue and tumor-flare reaction (referred to as TFR), and initial dose was reduced to 10 mg, with the possibility of daily dose increasing by 5 mg every cycle, based on treatment tolerance, up to a maximum of 25 mg[52]. Treatment was administered until PD or up to 2 years for patients who achieved a stable disease (SD) or PR, while patients in CR received two additional cycles before discontinuation. All patients received a median of six previous therapies (range: 1-14). In an intention-to-treat analysis, ORR was 28% (9/32 cases), all were PRs, with a median DOR of 10 mo [52]. The median TTR was 4 mo and 2 mo for an initial dose of 10 mg and 25 mg, respectively. Treatment response was achieved in a significant proportion of patients with blood or lymph node involvement and/or erythrodermic disease. Median PFS and OS were 8 mo and 43 mo, respectively; the main cause of death was PD (12/18 patients)[52]. Interestingly, immunophenotypic analysis showed lenalidomide effect could be associated with a reduction in circulating CD25-positive and CD4-positive T-cell count[52].

Nodal TCLS

The category of nodal PTCL includes angioimmunoblastic (AITL), anaplastic (ALCL) and PTCL not otherwise specified (NOS). PTCL-NOS represents the most common subtype, followed by AITL and ALCL (further divided in ALK-positive and ALK-negative ALCL)[1,2].

ALCL is characterized by a strong CD30 expression and large-cell anaplastic morphology; most ALK-positive cases occur in young adults and have a good prognosis, while ALK-negative cases are mainly reported in older population and display an aggressive behavior, with frequent disease relapse and/or chemorefractoriness[53]. AITL has peculiar pathophysiological features, such as the presence of an increased vascular proliferation and a reactive microenvironment (eosinophils, plasma

cells, epithelioid cells) in the lymph node, together with neoplastic T-cells[54]. Skin rash, B symptoms, hemolytic anemia and polyclonal hypergammaglobulinemia are frequently reported. Moreover, a deregulated T-cell response has been observed, with immune dysfunction[54]. A new category of neoplasms with derivation from CD4-positive T follicular helper (TFH) cells was defined in the last World Health Organization classification, which includes AITL, follicular TCL, and nodal lymphoma with a TFH phenotype[2]. PTCL-NOS is a heterogeneous subgroup of TCL cases, which do not fulfill the characteristics of more specific categories[1]. Due to this background, nodal PTCL, especially those with a TFH cell derivation, could represent an attractive target for lenalidomide.

To our knowledge, the first published study focused on lenalidomide for PTCL therapy is the interim report of a phase II trial by Dueck *et al*[55]. Both newly diagnosed (with contraindications to chemotherapy) and R/R TCL other than CTCL were enrolled from September 2006 to November 2008. The primary endpoint was ORR; patients received lenalidomide 25 mg daily on days 1-21 of a 28-d cycle, until PD or unacceptable side effects. Out of 24 patients, 20 were R/R; the diagnosis was PTCL-NOS (10 cases), AITL (7 cases), ALCL (5 cases), enteropathic-type and hepatosplenic γδ TCL (1 case each). The median number of previous regimens was 1 (range: 0-4), and 5/24 cases were refractory to last previous line. Out of 23 evaluable patients, ORR was 30% (all achieved a PR), and median PFS and OS were 96 d and 241 d, respectively (for patients with at least a SD, median PFS was 168 d)[55]. Among different histologies, ORR was 40% 29% and 33% for ALCL, AITL and PTCL-NOS, respectively. Interestingly, 2 PR patients were refractory to their previous regimen, and the development of skin rash was associated with treatment response[55].

All final reports of clinical trials focused on lenalidomide treatment for nodal TCL are illustrated in Table 2. Between November 2008 and June 2009, Zinzani *et al*[56] enrolled 10 R/R PTCL-NOS patients with stage II-IV disease in a prospective, single-arm, phase II trial. Lenalidomide initial dose was 25 mg for 21 d of a 28-d cycle for four cycles as induction phase. At disease restaging after the 4th cycle, patients who achieved at least a SD continued lenalidomide administration for another eight cycles with the same schedule as maintenance phase. The results after induction phase, with an ORR of 30% (3/10 cases, all CRs) and 1 SD, were encouraging, considering that most of patients were heavily pretreated (median number of 4 Lines, range of 2-7)[56]. After maintenance phase, CR was confirmed for all 3 patients, with 2/3 cases relapsed after 3 and 5 mo, respectively, while the remaining case maintained a durable CR at last follow-up after 11 mo. The patient with SD received all 12 cycles but developed PD 1 mo upon completion of the last treatment administration[56].

On 2013, the results of the above mentioned phase II EXPECT trial were published. Even if the primary end-point was met, the study was terminated early after 54 patients were enrolled, because the efficacy was considered unsatisfactory when compared to the results obtained with other investigated compounds[51]. Patients were heavily pretreated (median number of 3 prior therapies, range of 1-11), histological subtypes included AITL (26/54 cases), PTCL-NOS (20/54 cases), ALCL (3/54 cases) and 5 cases with extranodal TCL (3 MF, 1 cutaneous ALCL and 1 TCL nasal-type). The median drug exposure time was 42 d, while the median treatment duration was 176 d for patients achieving at least a PR[51]. ORR was 22% and CR rate was 11%; among the different histologies, ORR was 20% and 31% for PTCL-NOS and AITL, respectively[51]. The median DOR was 3.6 mo (not reached for patients in CR) and the response rate was slightly higher for younger *vs* older cases (27% and 18%, respectively). Median PFS for the entire cohort was 2.5 mo, while it was 4.6 mo and 1.9 mo for AITL and non-AITL cases, respectively; OS data were not shown[51].

On 2015, the final report of the above mentioned interim analysis was published by Toumishey *et al*[57]. Out of 39 enrolled patients (8 treatment-naïve cases), diagnosis was PTCL-NOS (14 cases), ALCL (10 cases), AITL (9 cases), lymphoblastic TCL, enteropathic-type and hepatosplenic γδ TCL (2 cases each). The median number of previous regimens was 1 (range: 0-5) and 11/39 cases were refractory to last previous line. ORR was 26% (10/39 cases), 3 patients achieved a CR. Among the different histologies, ORR was 10%, 33% and 43% for ALCL, AITL and PTCL-NOS, respectively [57]. The median PFS, DOR and OS were 4 mo, 13 mo and 12 mo, respectively. Interestingly, a trend between skin rash and treatment response was reported. When we analyzed newly diagnosed and R/R patients separately, ORR, median OS, PFS and DOR were 50% and 24%, 22 mo and 12 mo, 2 mo and 4 mo, 21 mo and 5 mo, respectively[57].

Due to the rarity of disease, we would like to mention several case reports about lenalidomide efficacy in R/R AITL. The peculiar pathophysiology of AITL makes the disease a suitable target for an immunomodulatory drug such as lenalidomide[58].

Our group administered lenalidomide to a patient with an unsatisfactory response despite three lines of chemotherapy. The patient received 12 treatment cycles (25 mg for four cycles and 15 mg for eight cycles) and maintained a durable CR after a follow-up of 30 mo[59]. In another report, a patient refractory to two previous lines received lenalidomide 15 mg continuously and achieved a CR, which was maintained after 2 years of follow-up[60]. Broccoli *et al*[61] administered reduced doses of lenalidomide (10 mg) to a refractory AITL patient with persistent disease and thrombocytopenia after autologous stem-cell transplantation. The patient achieved a long-lasting CR and continued therapy at an escalated dose of 15 mg for a total of 11 cycles. Finally, an 87-year-old woman with AITL and concurrent MM was refractory to multiple lines of chemotherapy and achieved a PR after four cycles of lenalidomide, with a dose escalation until 20 mg[62]. Interestingly, both AITL and MM improved after lenalidomide administration.

SAFETY

Lenalidomide is generally administered as an outpatient regimen and is well tolerated. As shown in Table 3, commonly observed side effects include neutropenia, thrombocytopenia, infections, skin rash and gastrointestinal disorders.

In the phase II study for R/R ATLL, the most common hematologic adverse event (AE) was thrombocytopenia (77%), while neutropenia and anemia occurred in 73% and 54% of total cases, respectively[44]. The incidence of grade 3-4 thrombocytopenia and neutropenia was 23% and 65%, respectively. The most frequent nonhematologic AEs were hypoalbuminemia (35%), constipation, hyponatremia and hypocalcemia (all with an incidence of 31%)[44]. Serious AEs were reported in 9 cases (35%), while AEs leading to treatment discontinuation were observed in 6 patients, including neutropenia and thrombocytopenia (2 cases each), toxic skin eruption (1 case), skin rash and hepatic failure (both in the same patient). Interestingly, no second primary malignancies (SPMs) were observed[44].

In the phase II trial focused on CTCL, the most common AEs were fatigue, lower leg edema and anemia, with an incidence of 59%, 47% and 41%, respectively[52]. Mild-to moderate constipation or diarrhea was observed in 11 cases (35%), while peripheral neuropathy was uncommon (19%). Grade 3-4 toxicity included fatigue (22%), infections (9%) and leukopenia (3%). Nine cases experienced a TFR, while no SPMs were reported[52].

In the Italian study, the incidence of grade 3-4 neutropenia, thrombocytopenia and asthenia was 25%, 15% and 10%, respectively[56]. In the EXPECT trial, grade 3-4 thrombocytopenia and neutropenia occurred in 20% and 15% of total cases, respectively[51]. Grade 3-4 infections and febrile neutropenia were observed in 15% and 4% of total cases, respectively; these events did not lead to treatment discontinuation. Grade 3-4 gastrointestinal disorders and TFR were experienced by 17% and 4% of total cases, respectively; only 1 patient had a treatment discontinuation due to TFR[51]. The most common AEs leading to treatment interruption or dose reduction were neutropenia and thrombocytopenia (11% of total cases each). Serious AE were reported in 54% of patients, the most frequently observed were hematological events and infections (19% of total cases each). SPMs were experienced by 3 patients, in 1 case the neoplasm was considered as therapy-related[51].

Finally, in the phase II trial by Toumishey *et al*[57], the most common AEs were pain (mainly considered as lymphoma-related rather than therapy-related), fatigue, gastrointestinal and hematological events[57]. The incidence of grade 3-4 anemia, neutropenia, febrile neutropenia, dyspnea, muscle weakness and dehydration was 11%, 16%, 8%, 13%, and 10%, respectively. Neutropenia was the main cause for dose reduction, while no deaths were considered as lenalidomide-related and no SPMs were reported[57].

NEW PERSPECTIVES

Lenalidomide monotherapy can produce a durable CR with manageable toxicity in R/R TCL, with an overall efficacy comparable to other investigated novel agents. New perspectives could be represented by combination strategies with lenalidomide in association with conventional chemotherapy and/or other novel agents, with the aim to have a place for earlier lenalidomide administration, even in a front-line regimen, especially for TCL with a TFH cell origin, such as AITL[56,57].

Table 3 Toxicity profile of lenalidomide in clinical trials for T-cell lymphomas

Toxicity/Adverse event	Ishida *et al*[44]	Querfeld *et al*[52]	Morschhauser *et al*[51]	Toumishey *et al*[57]
Hematological toxicity				
Anemia total	54%	41%	NR	26%
Anemia grade 3-4	19.2%	/	4%	11%
Leukopenia total	50%	22%	NR	NR
Leukopenia grade 3-4	38.5%	3%	4%	NR
Neutropenia total	73%	NR	NR	18%
Neutropenia grade 3-4	65.4%	NR	15%	16%
Thrombocytopenia total	77%	NR	NR	26%
Thrombocytopenia grade 3-4	23.1%	NR	20%	5%
Hypoalbuminemia	35%	28%	NR	NR
Grade 3-4	/	/	NR	NR
Constipation	31%	34%[1]	17%	44%
Grade 3-4	/	/	NR	3%
Nausea	23.1%	13%	NR	28%
Grade 3-4	3.8%	/	NR	/
Vomiting	23.1%	NR	NR	10%
Grade 3-4	/	NR	NR	/
Skin rash	23.1%	25%	NR	38%
Grade 3-4	7.6%	/	9%	11%
Fatigue	15.4%	59%	NR	56%
Grade 3-4	3.8%	22%	NR	11%
Diarrhea	NR	NR	NR	31%
Grade 3-4	NR	NR	NR	8%
Pain	NR	34%	NR	64%
Grade 3-4	NR	/	NR	21%
Infection	19.2%	34%	NR	26%
Grade 3-4	10.4%	9%	15%	5%
Neuropathy	NR	19%	NR	NR
Grade 3-4	NR	/	NR	NR
Lower leg edema	NR	47%	NR	28%
Grade 3-4	NR	/	NR	3%
Anorexia	NR	16%	NR	28%
Grade 3-4	NR	/	NR	5%
Respiratory disorders	10.4%	NR	NR	26%
Grade 3-4	7.6%	NR	13%	13%
Pulmonary embolism	NR	NR	NR	10%
Grade 3-4	NR	NR	NR	8%
Tumor flare reaction	NR	28%	14%	NR
Grade 3-4	NR	NR	4%	NR

[1]Considered together with diarrhea; Gastrointestinal disorders. NR: Not reported.

The genomic TCL landscape is being elucidated and a significant proportion of ATLL cases have shown alterations in interferon regulatory factor 4 and RHOA, which are involved in the mechanism of action of lenalidomide[63]. Due to the different mechanism of action, a combination regimen with mogamulizumab appears very promising[44-46]. The NK cells function is enhanced with lenalidomide, which could allow mogamulizumab to work better through an improvement of the antibody-dependent cell-mediated cytotoxicity[46].

The REVAIL study investigated lenalidomide in association with CHOP in newly diagnosed AITL patients. At the last International Conference on Malignant Lymphoma meeting, an ancillary study was presented in which bone marrow involvement (BMI), but no blood involvement, showed an association with reduced survival. Median PFS and OS for patients with or without BMI were 9 mo and 36 mo and 17 mo and 54 mo, respectively. The prognostic index for PTCL, including BMI, had the best power to divide the entire cohort between high-risk and low-risk cases, with a 2-year OS of 38% *vs* 79%. Moreover, BMI was associated with the presence of IDH-2 mutations[64].

An integrative analysis of this trial has been recently published, lenalidomide was given in association with CHOP every 21 d for eight total cycles[65]. CHOP was administered on day 1, and lenalidomide was added at a daily dose of 25 mg for 14 d every 21 d. Out of 78 patients included in the efficacy analysis, ORR was 56%, with a CR rate of 41%. The median dose intensity for lenalidomide was 81%, and 55% of total cases completed the study; the most common reasons for early treatment discontinuation were PD and toxicity[65]. After a median follow-up of 45 mo, 2-year PFS and OS were 42.1% and 59.2%, respectively; the presence of DNMT3A mutation appeared related with shorter PFS. Unfortunately, the primary end-point to improve a positron emission tomography-based CR rate from 45% to 60% was not reached, and the authors suggest the lack of benefit from adding lenalidomide to CHOP as AITL first-line therapy[65]. In 2 refractory AITL cases, lenalidomide was investigated in association with bortezomib and dexamethasone, achieving a CR and a PR with a manageable safety profile[66]. The rationale could be represented by a potential synergism between the immunomodulatory and antiangiogenic action of lenalidomide and the proteasome inhibitor activity of bortezomib.

In another phase I/II study, a combination of lenalidomide, vorinostat and dexamethasone was explored. Out of 8 enrolled R/R nodal TCL patients, 2 experienced a dose-limiting toxicity with a daily dose of 10 mg; thus, the maximum tolerated dose of lenalidomide was 5 mg/d[67]. ORR was 25% (1 CR and 1 PR), with a median PFS and OS of 2.2 and 6.7 mo, respectively. Due to these disappointing results, the authors did not find any additional benefit for this combination regimen compared to lenalidomide alone and discouraged further investigations[67].

The histone deacetylase inhibitor romidepsin showed a potential synergism with lenalidomide and could enhance tumor cell death in a TCL preclinical model[68]. This combination demonstrated a synergistic effect in the Hut-78 human TCL cell line and an additive effect in the Karpas-299 human TCL cell line; it was mainly related to the activation of a caspase-dependent pro-apoptotic pathway[68]. A phase Ib/IIa study, in which both PTCL and MM will be enrolled, is currently ongoing (NCT01755975).

Another promising strategy is represented by the use of lenalidomide as maintenance after debulking therapy, as previously published for R/R DLBCL cases[69]. Lenalidomide maintenance *vs* observation for advanced CTCL was investigated in a phase III, randomized trial at a daily dose of 25 mg for 21 d every 28 d[70]. Unfortunately, the trial was terminated early, following withdrawal of funding; out of 21 patients, 9/21 and 12/21 cases had been randomized to lenalidomide and observation. Median PFS was 5.3 mo and 2 mo, respectively, further suggesting a potential benefit for maintenance therapy, even if the reduced sample size did not permit a statistical comparison[70].

CONCLUSION

R/R TCL cases are characterized by poor prognosis and current guidelines showed a lack of satisfactory treatment options. Lenalidomide has been successfully used in several hematologic malignancies, such as mantle-cell lymphoma, MM, DLBCL and myelodysplatic syndromes. The multiple mechanisms of action, with immunomodulatory, antiangiogenic and direct antineoplastic properties, represent a strong rationale to investigate the drug, alone or in association, for the treatment of R/R TCL patients. Lenalidomide demonstrated a promising efficacy with manageable toxicity in the

treatment of ATLL, CTCL and nodal TCL, at least comparable to licensed drugs such as mogamulizumab, pralatrexate or romidepsin, even in heavily pretreated patients. Identification of the TFH cell as the cell of origin of several TCLs, including AITL, could explain the high efficacy of lenalidomide in the treatment of R/R AITL cases. To our knowledge, combination strategies did not show an additional benefit compared to monotherapy, even if further investigations are warranted.

In conclusion, lenalidomide as a single-agent prolongs PFS in R/R TCL with an acceptable toxicity and could represent a suitable treatment option for this patient population, especially for neoplasm cases with a TFH origin.

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MINIREVIEWS

Current update on imaging for pancreatic neuroendocrine neoplasms

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Abstract

Pancreatic neuroendocrine neoplasms (panNEN) are a heterogeneous group of tumors with differing pathological, genetic, and clinical features. Based on clinical findings, they may be categorized into functioning and nonfunctioning tumors. Adoption of the 2017 World Health Organization classification system, particularly its differentiation between grade 3, well-differentiated pancreatic neuroendocrine tumors (panNET) and grade 3, poorly-differentiated pancreatic neuroendocrine carcinomas (panNEC) has emphasized the role imaging plays in characterizing these lesions. Endoscopic ultrasound can help obtain biopsy specimen and assess tumor margins and local spread. Enhancement patterns on computed tomography (CT) and magnetic resonance imaging (MRI) may be used to classify panNEN. Contrast enhanced MRI and diffusion-weighted imaging have been reported to be useful for characterization of panNEN and quantifying metastatic burden. Current and emerging radiotracers have broadened the utility of functional imaging in evaluating panNEN. Fluorine-18 fluorodeoxyglucose positron emission tomography (PET)/CT and somatostatin receptor imaging such as Gallium-68 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid–octreotate PET/CT may be useful for improved identification of panNEN in comparison to anatomic modalities. These new techniques can also play a direct role in optimizing the selection of treatment for individuals and predicting tumor response based on somatostatin receptor expression. In addition, emerging methods of radiomics such as texture analysis may be a potential tool for staging and outcome prediction in panNEN, however further investigation is required before clinical implementation.

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Grade D (Fair): 0
Grade E (Poor): 0

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Core Tip: Imaging plays a critical role in the diagnosis and management of pancreatic neuroendocrine neoplasms. Enhancement patterns and diffusion-weighted imaging aid the detection and classification of these lesions. Contrast-enhanced magnetic resonance imaging is useful for the evaluation of hepatic metastases. Dual-tracer positron emission tomography/computed tomography with Gallium-68 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid–octreotate and Fluorine-18 fluorodeoxyglucose may be particularly useful for distinguishing grade 3 pancreatic neuroendocrine tumor from pancreatic neuroendocrine carcinoma. Furthermore, these advanced imaging techniques can help in the staging and detection of distant metastases. Evaluation of somatostatin receptor expression and metabolic activity with functional imaging can help select optimal treatment.

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INTRODUCTION

Pancreatic neuroendocrine neoplasms (panNEN) represent a rare, diverse group of neoplasms[1]. These tumors account for less than 2% of pancreatic cancers and only 7% of all neuroendocrine tumors. These entities can manifest at any age but are most often diagnosed in individuals between 40 and 65 years old. The majority of panNEN are sporadic[2]. Up to 10% are associated with hereditary disorders including Von Hippel-Lindau disease, neurofibromatosis type 1, tuberous sclerosis complex, and multiple endocrine neoplasia type 1 (MEN1) syndrome, which increase a patient's predilection for neoplasms. PanNEN can be categorized into functioning and nonfunctioning neoplasms based on clinical findings. Recent discoveries on the mechanisms behind panNEN pathogenesis and molecular cytogenetics have resulted in significant changes regarding their classification, diagnosis, and treatment. In particular, new distinctions in classification between well-differentiated pancreatic neuroendocrine tumors (panNET) and poorly-differentiated pancreatic neuroendocrine carcinomas (panNEC) has emphasized the need for more advanced imaging techniques to guide diagnosis and follow-up[1]. In this review, we will discuss the most current classifications of panNEN based on pathology, genetic, and clinical features. In addition, we will review the use of anatomic imaging modalities like ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI) for initial detection and management, along with molecular imaging techniques that have proven useful for identifying occult tumors and further characterization. The potential use of CT, MRI, and positron emission tomography (PET)/CT texture analysis to grade tumors and predict clinical outcome will also be briefly highlighted.

PATHOLOGY

PanNEN demonstrate two histopathological classifications: panNET and panNEC. PanNET account for more than 90% of panNEN and are characterized as well-differentiated neoplasms that manifest with little to moderate atypia. On gross examination, they appear well-circumscribed by a thin capsule. Cystic changes and hemorrhage may be identified. PanNEC can manifest as a small cell or large cell variant. The large cell variation comprises 60% of panNEC and exhibits expansile growth. Small cell panNEC exhibit more infiltrative growth. Necrosis and vascular invasion are com-

monly observed[3].

The 2010 World Health Organization (WHO) classification system for panNEN based categorization on a neoplasm's Ki-67 proliferation index and mitotic index. In this system, when both indices are greater than 20, the tumor is classified as panNEC. Subsequently, many large studies showed the existence of well-differentiated panNET presenting high mitotic and Ki-67 indices. Thus, the 2017 WHO classification system (Table 1) accounts for both the level of proliferation and differentiation of neoplasms, distinguishing a well-differentiated grade 3 panNET from a poorly-differentiated grade 3 panNEC[1,4]. Additional changes include the renaming of mixed adenoneuroendocrine carcinomas to mixed neuroendocrine-nonneuroendocrine neoplasms (MiNEN), in order to reflect their capacity to manifest not only as high-grade, malignant neoplasms, but also as low-grade, benign tumors. MiNEN are composed of both neuroendocrine and non-neuroendocrine components and have relatively non-specific features, tending to mimic panNEC[1].

Although WHO classification relies on pathological features to distinguish grading, single location biopsy may not be an accurate representation of all tumor burden due to the variance within and between lesions. In addition, grade transformation can occur following biopsy. Thus, imaging evaluation and follow-up often play an important role in dictating ongoing and future management, regardless of initial grading.

MOLECULAR CYTOGENETICS

Research focusing on the study of panNEN pathogenesis has significantly broadened the knowledge behind genetic mutations which may influence these lesions and their prognosis. The most common genetic alterations seen in panNET include mutations of the tumor suppressor gene *MEN1*, and chromatin-remodeling genes *ATRX* and *DAXX* [3]. *MEN1* encodes the protein menin, which is involved in histone methylation and cell cycle inhibition. *MEN1* mutations are seen in 31% to 44% of grade 3 panNET, resulting in the disruption of tumor suppression[5]. The majority of these mutations are sporadic, but some may be inherited and seen in association with MEN1 syndrome, Von Hippel Lindau syndrome, neurofibromatosis type 1 and tuberous sclerosis. *ATRX* and *DAXX* mutations are strongly associated with high grade tumors and poor outcomes. A mutation in one of the two genes is observed in more than 45% of well-differentiated neoplasms, and result in an alternative lengthening of telomeres phenotype which correlates with aggressive behavior. *DAXX* abnormalities are also associated with low expression of *TP53*, a tumor suppressor gene that is involved in apoptosis, cell proliferation, and DNA repair. Other molecular abnormalities that may be observed in panNET are mutations in *TSC1* and *TSC2*, *PTEN*, *PIK3CA*, and *DEPDC5*, which all play a role in the mammalian target rapamycin (mTOR) pathway. These mutations occur in approximately 15% of tumors[1,3].

The molecular abnormalities driving panNET do not usually occur in panNEC. Instead, these neoplasms commonly feature mutations in *TP53* and *Rb1*. *KRAS* and *SMAD4* mutations can also occur, but these are less frequent[1].

CLINICAL FEATURES

PanNEN have a wide range of clinical findings, depending on the subtype. The clinical presentation of functioning panNET is influenced by their characteristic hypersecretion of various hormones. Insulinomas account for 60% of functioning panNET and are composed of insulin-producing β cells[3]. They typically manifest with Whipple's Triad (*i.e.* fasting hypoglycemia, symptoms of hypoglycemia, and relief of symptoms following administration of IV glucose)[2]. About 10% of cases will present multiple insulinomas, usually in association with MEN1 syndrome. Gastrinomas represent the second most common functioning panNET. They usually arise in the gastrinoma triangle, a region enclosed by the pancreatic head and neck, the second and third part of the duodenum, and the cystic and common bile duct[1]. Overproduction of gastrin leads to the onset of Zollinger-Ellison syndrome, resulting in peptic ulcer disease, secretory diarrhea, or gastroesophageal reflux disease[3]. Glucagonoma is characterized by its hypersecretion of glucagon. Common manifestations include necrolytic migratory erythema, diabetes mellitus, deep vein thrombosis, and depression[3,6,7]. Other functioning panNET are somatostatinomas, vasoactive intestinal peptide-secreting tumors, and adrenocorticotropic hormone-secreting tumors, which comprise

Table 1 Comparison of 2010 and 2017 World Health Organization classification system for pancreatic neuroendocrine tumors

WHO 2010 Classification system	WHO 2017 Classification system	Ki-67 index (%)	Mitotic index[1]
Well-differentiated PanNET G1	Well-differentiated PanNET G1	< 3	< 2
Well-differentiated PanNET G2	Well-differentiated PanNET G2	3-20	2-20
	Well-differentiated PanNET G3	> 20	> 20
Poorly-differentiated PanNEC G3 (*i.e.* small cell carcinoma, large cell carcinoma)	Poorly-differentiated PanNEC G3 (*i.e.* small cell carcinoma, large cell carcinoma)	> 20	> 20
MiNEN	MANEC		

[1]Per 10 high-power fields. WHO: World Health Organization; PanNEN: Pancreatic neuroendocrine neoplasms; PanNET: Pancreatic neuroendocrine tumors; PanNEC: Pancreatic neuroendocrine carcinomas; MiNEN: mixed neuroendocrine-nonneuroendocrine neoplasms; MANEC: Mixed adenoneuroendocrine carcinomas.

less than 20% of cases[8].

Nonfunctioning panNET are usually asymptomatic until advanced stages, resulting in later presentation and diagnosis. These tumors can secrete polypeptides; however, such secretions do not lead to any associated clinical findings. When symptoms do appear, they are often a result of tumor burden and its mass effect. Up to 50% of nonfunctioning panNET present distant metastases, particularly in the liver, although other locations include the lungs, bone, peritoneum, adrenal glands, brain, and spleen [3]. Similarly, metastatic disease is a common clinical feature of panNEC. A retrospective study reported 88% of panNEC in their cohort demonstrated metastases upon diagnosis[9].

IMAGING FEATURES

Imaging plays a critical role in diagnosing and evaluating panNEN. Conventional modalities like US, CT, and MRI are often used in the initial detection of panNEN. Techniques using PET/CT and novel radiotracers have proven to be extremely useful in the identification and classification of these tumors.

US

On sonography, panNEN usually appear as a well-defined, solid, heterogeneous hypoechoic mass (Figure 1). Some lesions may present with cystic regions[8,10]. Hepatic metastases from panNEN are often hyperechoic in comparison to surrounding liver parenchyma, however they can also manifest as hypoechoic and targetoid lesions. Doppler US reveals increased vascularity. Endoscopic US (EUS) is the preferred modality for detecting small, occult panNEN that are difficult to see with noninvasive techniques[1]. EUS has been reported to have 80% to 90% sensitivity towards panNET, including those that remain undetected on CT and transabdominal US[11-14]. EUS sensitivity towards small insulinomas and duodenal gastrinomas is particularly useful, as these lesions can often be overlooked by other modalities. Following microbubble contrast, panNET show early, intense enhancement on EUS, differentiating these tumors from panNEC or pancreatic ductal adenocarcinoma (PDAC) which are generally hypovascular. Homogeneous enhancement typically indicates a lower Ki-67 index[1]. Other benefits of EUS include its capacity for tissue acquisition using fine needle aspiration or core biopsy; EUS-guided biopsies agree with surgical Ki-67 evaluation in up to 84% of cases[15-18]. Intraoperative US also plays a useful role in some cases by allowing for accurate localization of neoplasms in relation to adjacent structure, thus reducing the risk of postoperative fistulas[1].

CT

CT is commonly used for initial assessment of suspected panNET. Given its high spatial resolution, CT provides excellent diagnostic information with regards to the detection and characterization of the primary tumor and allows assessment of local vascular spread and distant metastatic spread. Typical CT protocol involves multiphasic imaging with pre-contrast acquisition and arterial, pancreatic, and venous phase acquisition following contrast[19]. Pre-contrast images may be useful in cases where there is hemorrhage. Following contrast administration panNEN are generally

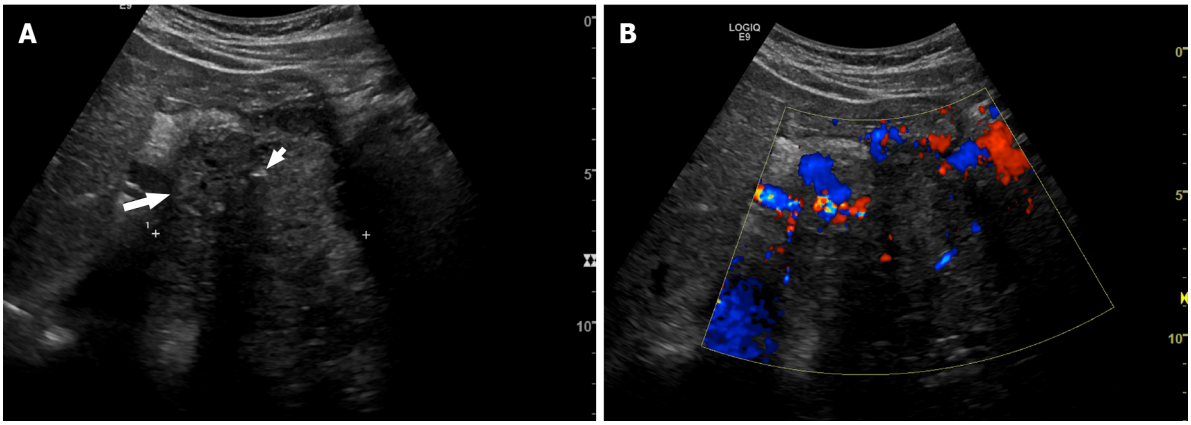


Figure 1 Forty-year-old man with pancreatic neuroendocrine neoplasm. A: Axial ultrasound shows a large solid heterogeneous mass (long arrow). Internal calcification (small arrow) is seen, causing posterior acoustic shadowing; B: Doppler ultrasound shows increased vascularity within the pancreatic tumor.

hyperenhancing (Figure 2) in comparison to surrounding pancreatic tissue on arterial phase and remain mildly hyperattenuating on venous and delayed phases. However, more subtle discrimination of enhancement patterns may allow further classification. Intense, homogeneous enhancement is typical of lower grade panNEN. Grade 1 and 2 neoplasms often appear as small, well-circumscribed lesions, best depicted on arterial phase. These tumors may contain cystic regions in up to 15%-20% of cases[20,21], and are more common in cases associated with MEN1. Pancreatic ductal dilation is more commonly seen in high-grade neoplasms and mixed tumors than well-differentiated panNEN; however, ductal dilation in low-grade tumors may be seen with secretion of serotonin. Grade 3 tumors are characterized as large, ill-defined masses that manifest with mild to low enhancement on arterial phase. They are typically hypointense on portal venous phase imaging. Heterogeneous attenuation due to necrosis and cystic change and the presence of lymphadenopathy or metastatic disease is common.

CT radiomics may be useful for distinguishing the grade of panNEN based on tumor heterogeneity and spatial variation when imaging findings are ambiguous. Texture analysis interprets the distribution of pixel values and position within an image to provide objective, quantitative evaluation of tissue heterogeneity. Guo *et al* [22] found texture parameters such as mean grey-level intensity, entropy, and uniformity demonstrated adequate sensitivity (73%-91%) and specificity (85%-100%) when differentiating grade 1 and 2 panNET from grade 3 panNEC, suggesting texture analysis may be useful for staging panNEN. Mean grey-level intensity showed up to a 100% sensitivity and 91% specificity for distinguishing grade 1 and grade 2 panNET. Canellas and colleagues reported significant differences between low-grade (grade 1) and high-grade (grade 2 and 3) panNEN in texture parameters including skewness, mean of positive pixels, and entropy. However, the only parameter that was an independent predictor of tumor grade was entropy. In addition, further investigation and standardization of postprocessing techniques is required before texture analysis can be applied in a clinical setting[23].

In conjunction with clinical findings, CT can also aid distinguishing functioning from nonfunctioning panNET. Functioning panNET tend to be smaller and more homogenous lesions. Gastrinomas may present ring-like enhancement. Nonfunctioning panNET are usually larger, heterogeneously enhancing masses, and are more likely to exhibit local or vascular spread. Necrosis, cystic changes, and calcifications may be observed[1,8]. Larger nonfunctioning panNET are more likely to exhibit aggressive behavior and often present with metastatic disease.

Hepatic metastases demonstrate intense enhancement on arterial phase imaging and only mildly enhance during the portal venous phase. Similar to gastrinoma, ring-like enhancement may also be seen and can be useful for differentiating panNEN-related metastatic disease from other hepatic lesions[1,11].

MRI

MRI provides improved detection of panNEN and hepatic metastases over abdominal sonography and CT given its superior contrast resolution (Figure 3). MRI enhancement patterns on arterial, venous, and delayed sequences are similar to those seen on CT. Fat-suppressed and diffusion-weighted imaging are particularly useful for identifying small, occult lesions and recognizing associated edema[11]. On MRI,

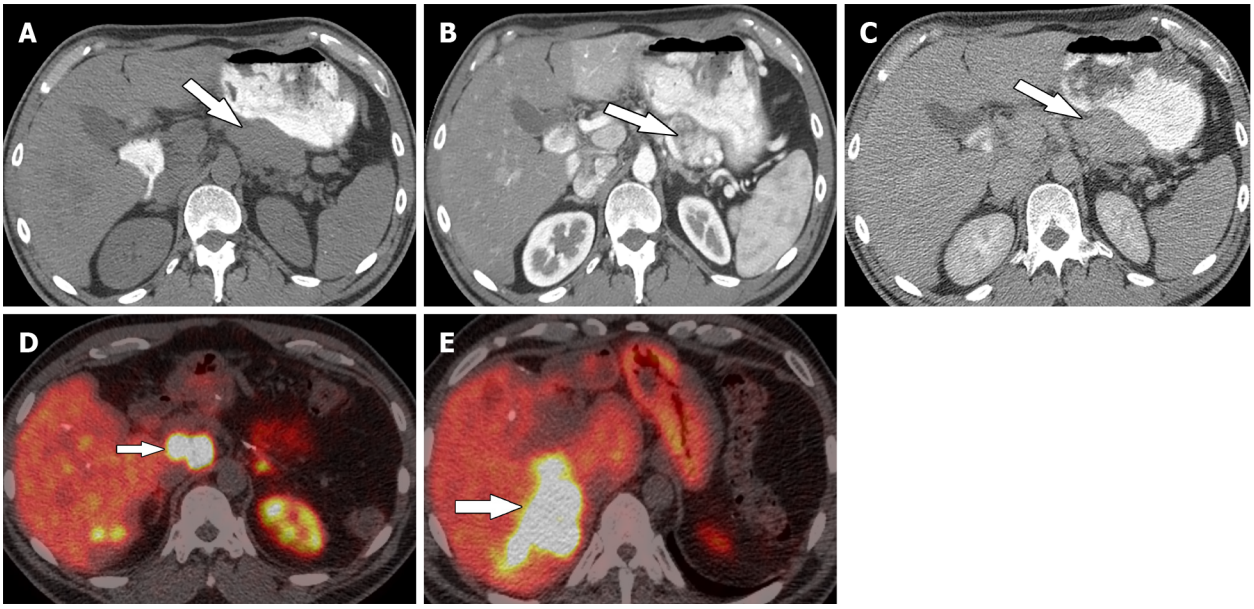


Figure 2 Thirty-eight-year-old woman with pancreatic neuroendocrine neoplasm. A: Axial precontrast computed tomography; B and C: Contrast-enhanced computed tomography in the arterial phase (B) and delayed phase (C) demonstrate pancreatic neuroendocrine neoplasm (arrow). Patient underwent surgical resection; D and E: Follow-up Gallium-68 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid–octreotate positron emission tomography/computed tomography shows metastatic adenopathy (short arrow) and liver metastases (long arrow).

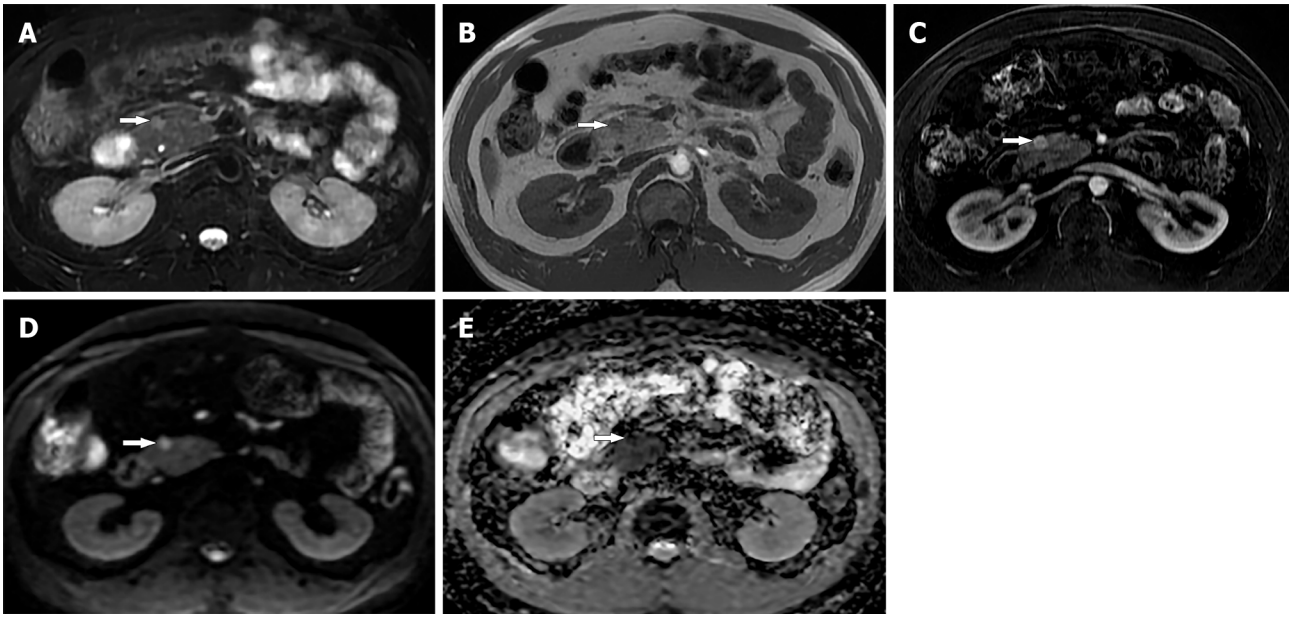


Figure 3 Thirty-five-year-old male with small pancreatic neuroendocrine neoplasm. A: Axial magnetic resonance T2 weighted image; B: T1 weighted image show a small 1 cm mass (arrow) in the head of pancreas; C: Arterial phase image shows avid enhancement in the tumor; D: Diffusion-weighted image; E: Apparent diffusion coefficient map show restricted diffusion within the tumor (arrow). Biopsy confirmed diagnosis of pancreatic neuroendocrine neoplasm.

panNEN typically manifest as hypointense on T1-weighted imaging and isointense on portal venous and delayed phases. Low-grade panNEN tend to exhibit high T2 signal while high-grade neoplasms typically exhibit low to intermediate hyperintensity on T2-weighted imaging[1].

Differentiating between panNEC and grade 3 panNET is challenging on imaging alone (Table 2). PanNEC usually share similar enhancement patterns to grade 3 panNET. Imaging features such as hypoenhancement or rim-like enhancement on arterial phase, persistent enhancement on portal venous phase, and hyperenhancement on delayed phase imaging may favor a diagnosis of panNEC over panNET. On diffusion-weighted imaging, panNEC also demonstrate high signal intensity and low apparent diffusion coefficient (ADC) in comparison to grade 3 panNET[1];

Table 2 Imaging features of grade 3 pancreatic neuroendocrine tumors *vs* grade 3 pancreatic neuroendocrine carcinomas

Grade 3 PanNET	Grade 3 PanNEC
Smaller, more defined lesions	Larger, ill-defined lesions
Absence of ductal dilation or metastatic disease	Ductal dilation or metastatic disease
Low to moderate homogeneous enhancement on arterial phase imaging	Heterogeneous or rim-like enhancement on arterial phase imaging
Hypointense on delayed phase imaging	Atypical persistence of enhancement on delayed phase imaging
Higher ADC values	Signal hyperintensity on diffusion-weighted MRI and lower ADC value
Low uptake on ^{18}F-FDG PET/CT	High uptake on ^{18}F-FDG PET/CT
Moderate uptake on ^{68}Ga-DOTATATE PET/CT	Low uptake on ^{68}Ga-DOTATATE PET/CT

MRI: Magnetic resonance imaging; ADC: Apparent diffusion coefficient; PanNET: Pancreatic neuroendocrine tumors; PanNEC: Pancreatic neuroendocrine carcinomas; ADC: Apparent diffusion coefficient; ^{18}F-FDG: Fluorine-18 fluorodeoxyglucose; PET/CT: positron emission tomography/computed tomography; ^{68}Ga-DOTATATE: Gallium-68 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid–octreotate.

however exact ADC cutoffs vary between studies and are not typically used in clinical practice to differentiate between panNEC and panNET[24-26]. The presence of ductal dilation and metastatic disease may indicate panNEC rather than panNET[1].

MRI is very helpful towards assessing the spread of panNEN to the liver[27]. Hepatic metastases are usually heterogeneously hyperintense on T2-weighted imaging, though atypical presentations include low to moderate T2 intensity. PanNEN hepatic metastases are typically hyperintense on the arterial phase of MRI. A peripheral ring of enhancement with gradual internal enhancement may also occur[1,11,28]. The apparent size of metastases can also vary depending on the dynamic contrast phase on which the dimension is measured. For estimation of tumor load, measurements on the hepatobiliary phase of gadoxetate MRI may be more accurate[29,30]. Histogram analysis of ADC maps could be useful for further indicating the aggressiveness and spread of panNEN. ADC entropy and kurotsis were reported to increase with tumor grade and vascular invasion. These parameters may also be useful for distinguishing panNEN with lymph node or distant metastasis, as both increase with the presence of metastases[31].

Functional imaging

The majority of panNEN express somatostatin receptors, allowing for excellent detection and characterization of these lesions using somatostatin analogs (SSA) coupled with radionuclide tracers. These techniques represent the forefront of panNEN imaging and can help to select patients for peptide receptor radionuclide therapy (PRRT)[1].

Somatostatin receptor scintigraphy (SRS) with Indium 111 (^{111}In)-pentetreotide can identify primary or metastatic disease throughout the body with 77% sensitivity and provides functional information on tumor somatostatin receptor expression[1,8]. However, SRS is limited due to its nonspecific uptake in other organs and inflammatory tissues. In addition, its poor spatial resolution and comparatively low affinity for somatostatin receptors has led to the adoption of substantially superior PET/CT techniques[32].

Gallium-68 (^{68}Ga) 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)–octreotate, more commonly called ^{68}Ga-DOTATATE, has demonstrated consistently high specificity (81%-100%) and sensitivity (90%-100%) as a PET agent for panNET[33,34] (Figure 4). ^{68}Ga-DOTATATE PET/CT is particularly useful for distinguishing low-grade, well-differentiated panNEN, which show greater ^{68}Ga-DOTATATE uptake than high-grade panNEN. Grade 3 panNET exhibit moderate uptake, while panNEC exhibit relatively poor uptake[1]. Physiological uptake in the pancreatic uncinate process is observed in up to one-third of individuals. The European Association of Nuclear Medicine (EANM) recommends disregarding uptake in the pancreatic uncinate process unless corresponding imaging findings are seen [35]. Other ^{68}Ga-DOTA-peptides include DOTATOC and DOTANOC, which are reported to have similar diagnostic yields as to ^{68}Ga-DOTATATE.

The decrease of somatostatin receptors seen in higher grade, less differentiated neoplasms is accompanied by an increase in metabolic activity, making Fluorine-18 fluorodeoxyglucose (^{18}F-FDG) PET an ideal technique for identifying these lesions. Grade 3 tumors have a reported median maximum standardized uptake value of 11.7

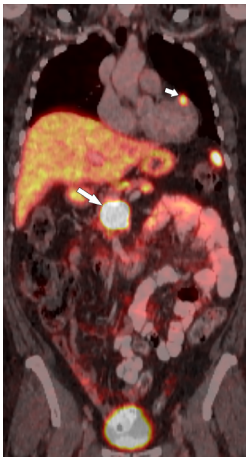


Figure 4 Sixty-two-year-old female with metastatic pancreatic neuroendocrine neoplasm. Coronal fused Gallium-68 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid–octreotate (DOTATATE) positron emission tomography/computed tomography shows a large soft tissue mass in the pancreatic head with intensely avid DOTATATE uptake. Note the subtle metastatic lesion in the pericardium (short arrow) along the left atrium.

for ^{18}F-FDG, *vs* 4.4 for ^{68}Ga-DOTATATE[1]. Conversely, tumors with a Ki-67 index lower than 10% showed minimal ^{18}F-FDG uptake, but high ^{68}Ga-DOTATATE uptake [36]. Dual-tracer PET/CT with ^{68}Ga-DOTATATE and ^{18}F-FDG may be useful for distinguishing grade 3 panNET from panNEC, as higher uptake of ^{68}Ga-DOTATATE indicates grade 3 panNET, while higher uptake of ^{18}F-FDG indicates panNEC[1,35]. The use of SSA-PET/CT combined with texture analysis may also be a useful indicator of prognosis. A multi-center retrospective study demonstrated higher entropy could predict greater overall survival[37].

A minority of insulinomas (< 10%) are negative on all conventional modalities due to their small size[35]. In such instances, SSA-PET/CT is a poor alternative, with a reported 25% sensitivity and specificity[38-43]. ^{18}F-dihydroxyphenylalanine (^{18}F-DOPA) PET/CT may aid localization of insulinomas, offering high sensitivity in cases of hyperinsulinemic hypoglycemia. However, this technique frequently results in positive findings for non-neuroendocrine pancreatic lesions and is only indicated for detecting non-pancreatic NENs by 2017 EANM guidelines[44]. Carbidopa premedication may increase ^{18}F-DOPA specificity towards insulinomas by inhibiting physiologic uptake. Multiple retrospective studies with small cohorts using ^{18}F-DOPA and carbidopa premedication have demonstrated insulinoma detection rates of 70-85% [45-47]. However, further investigation into the role of ^{18}F-DOPA PET/CT in panNEN is required.

Glucagon-like peptide receptor (GLP-1R) PET/CT may also prove useful for detecting insulinomas. The majority of benign insulinoma express GLP-1R, resulting in a sensitivity on GLP-1R -based PET/CT of more than 95%[47,48]. However, uptake in the pancreatic tail can be mistaken for physiological renal accumulation of radionuclides; uptake by duodenal Brunner gland may be mistaken for an insulinoma in the pancreatico-duodenal groove. In addition, malignant insulinomas express GLP-1R considerably less than their benign counterparts [35,49].

DIFFERENTIAL DIAGNOSIS

The differential diagnosis for panNEN includes other hypervascular pancreatic lesions. Pancreatic metastases from renal cell carcinoma, melanoma, and sarcoma may often appear as hypervascular masses resembling panNEN. In particular, renal cell carcinoma may present with late onset metastasis in pancreas, even 5 to 10 years following treatment of the primary tumor, causing diagnostic dilemma. A history of previous primary malignancies should alert to the possibility of pancreatic metastases over panNEN. Serous cystadenomas represent another possible mimic of panNEN, particularly the rare subset of cases which may appear solid on CT. T2-weighted usually reveals presence of multiple septated cysts in serous cystadenomas which may occasionally not be apparent on CT. Lack of uptake on ^{68}Ga-DOTATATE PET/CT is also useful for separating serous cystadenomas from panNEN. Intrapancreatic accessory splenules in the pancreatic tail may be another potential pitfall causing

diagnostic confusion, especially if only a single-phase CT is available. However, on MRI this diagnosis is generally straightforward. Splenules will have the same appearance as normal spleen on all MR sequences including T1-weighted, T2-weighted, diffusion-weighted and postcontrast sequences. In cases of diagnostic difficulty, uptake on technetium 99m (^{99m}Tc)-labeled heat-damaged red blood cell-tagged or ^{99m}Tc-labeled sulfur colloid scans may help. Cystic panNEN may be mistaken for other cystic pancreatic entities such as mucinous cystic neoplasms, in which case EUS-guided fine needle aspiration might be necessary to confirm diagnosis [1].

Distinguishing the typical well-differentiated panNET from PDAC is usually straightforward, as panNET typically are hypervascular, well-defined and do not typically cause ductal obstruction. Nevertheless, the imaging appearance of panNEC often overlap with PDAC given their shared hypovascularity and ill-defined borders. These similar radiologic findings may result in misdiagnosis of up to 57% of panNEC as PDAC[31]. Decreased portal phase enhancement and a lower enhancement ratio between arterial and portal phase may raise suspicion for PDAC over nonhypervascular panNEN[50-52]. Features that are more common in panNEC include tumoral calcification and vascular invasion[1,31]. CT texture analysis may be useful as panNEC typically demonstrate more intratumoral homogeneity than PDAC. Consequently, panNEC demonstrate higher uniformity and lower entropy than PDAC at portal phase imaging[50]. Texture analysis based on ADC values may also improve diagnostic capabilities; ADC histogram analysis of diffusion-weighted imaging revealed PDAC demonstrate higher kurtosis and skewness on ADC_{400} and ADC_{800} than panNEN, overall. PanNEN exhibited significantly lower entropy regardless of *b* value [31]. However, definitive discrimination between panNEC and PDAC using imaging alone is difficult, and histological diagnosis is usually warranted.

MANAGEMENT

The management of panNEN varies with their classification and the degree of local and metastatic spread. Localized, asymptomatic panNET less than 2 cm in size are usually treated conservatively with active surveillance[53]. However, larger or symptomatic panNEN require more comprehensive treatment such as symptom-directed therapy, SSA therapy, molecularly-targeted and conventional chemotherapy, or peptide receptor radionuclide therapy. Liver-specific therapy may be used to treat hepatic metastases[54,55].

Surgical resection and debulking

Surgical resection is currently used for nonfunctioning tumors larger than 2 cm, and functioning panNET of any size. Accurate tumor localization is critical for operative success. ^{68}Ga-DOTATATE PET/CT is the preferred imaging study for evaluating the spread of noninsulinoma panNET[54]. Selective arterial calcium stimulation with hepatic venous sampling is occasionally used to localize insulinomas that are difficult to assess on anatomic imaging. The emergence of GLP-1R PET/CT represents a superior alternative to this technique[54,56,57]. Simple enucleation may be sufficient for smaller, low-grade tumors that are at least 2-3 mm away from the main pancreatic duct. MR cholangiopancreatography and EUS are useful for estimating this distance [54]. The majority of functioning panNET require more extensive resection and lymphadenectomy[1]. A total pancreatectomy may be considered for multifocal disease. Noncurative surgical debulking may be pursued in cases of unresectable, metastatic panNEN to palliate symptoms and extend survival[1]. However, advanced disease is typically managed with non-surgical treatment strategies (Figure 5).

SAA therapy

SSA such as octreotide or lanreotide is often used in the management of advanced, progressive tumors. In addition to their antisecretory benefits these drugs have cytostatic effects on the tumor, as proven by the multicenter, phase III CLARINET and PROMID trials which demonstrated an increase in estimated progression-free survival [58,59]. However, this effect appears to be diminished in tumors that do not show adequate uptake on somatostatin imaging techniques. Koch *et al*[60] reported a 2.9-fold increased probability of achieving stable disease following SSA therapy in neuroendocrine tumors with high uptake on ^{68}Ga-DOTATATE PET, in comparison to tumors with low uptake. Thus, a multidisciplinary panel of experts convened by the Society for Medicine and Molecular Imaging (SNMMI) suggested the potential utility of ^{68}Ga-DOTATATE PET/CT in selecting patients with nonfunctioning panNET for soma-

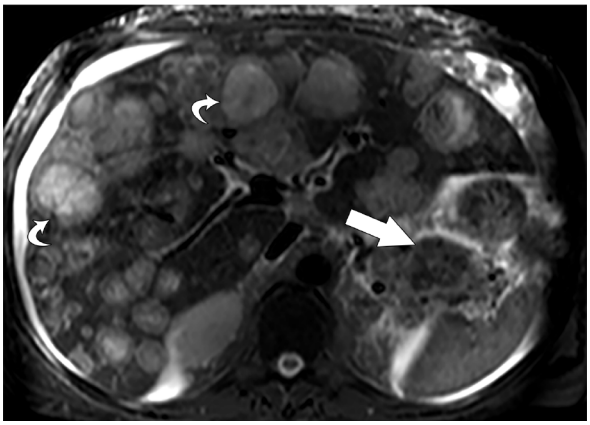


Figure 5 Thirty-nine-year-old male with metastatic pancreatic neuroendocrine neoplasm. Axial T2 weighted image shows innumerable bilobar metastases (curved arrows). Note the heterogeneous primary pancreatic neuroendocrine tumor (straight arrow). Patient was treated with capecitabine and temozolomide.

tostatin analog therapy. However, the SNMMI expert panel agreed that in the case of symptomatic manifestations, SSA therapy is indicated regardless of imaging findings [61].

Molecularly targeted chemotherapy

Molecularly targeted chemotherapy using agents such as everolimus, an mTOR inhibitor, and sunitinib, a tyrosine kinase inhibitor, have been reported to improve progression-free survival of individuals with grade 3 panNET and metastatic disease [1]. Early studies on emerging agents including multi-targeted kinase inhibitors and a combination of temsirolimus and bevacizumab, also show positive results[55,62].

Conventional chemotherapy

Although SSA and molecular therapies have shown significant benefits in patients with panNEN, conventional chemotherapy or PRRT is preferred for highly symptomatic patients and those with rapidly growing metastases. A streptozocin-based regimen or a combination of temozolomide and capecitabine is the optimal approach for panNET[55]. Platinum-based chemotherapies such as cisplatin with etoposide or irinotecan are the regimen of choice for panNEC, with reported response rates of 60% [1,63].

Peptide receptor radionuclide therapy

Peptide receptor radionuclide therapy (PRRT) uses SSA to deliver radionuclides such as yttrium-90 (^{90}Y) and lutetium-177 (^{177}Lu). These agents deliver beta radiation or high energy electrons, causing localized cellular necrosis at the site of accumulation, and have been associated with promising outcomes in grade 1 and 2 panNET. One phase II, single-center clinical trial demonstrated an increase in median survival by 26 mo in neuroendocrine tumor patients treated with PRRT[64-68]. However, PRRT may be less useful in panNEC due to their lower somatostatin receptor expression[1]. In addition, panNET with lower expression of somatostatin receptors may be susceptible to a similar decrease in response rate. Multiple studies propose the use of the "NETPET" scoring system developed by Chan and colleagues, and similar PET/CT-dependent classification, to select patients for PRRT[69-72]. In the NETPET system, tumors are graded from P1 to P5 based on their avidity on ^{68}Ga-DOTATATE and ^{18}F-FDG PET, with a score of 1 indicating positive results on ^{68}Ga-DOTATATE but not ^{18}F-FDG PET, and a score of 5 indicating positive results on ^{18}F-FDG PET but not on ^{68}Ga-DOTATATE PET. However, further investigation into the correlation between PRRT outcome and NETPET scores must be done to establish if such imaging-based classification systems have a role in clinical settings. Other methods for predicting PRRT response include the measurement of skewness and kurtosis based off ^{68}Ga-DOTATATE imaging; Önner *et al*[73] reported significantly higher skewness and kurtosis in tumors which did not response to treatment that those that did. Nevertheless, the diagnostic ability of the two metrics to indicate poor PRRT response remained moderate to low.

Liver-specific therapy

In the presence of hepatic metastases, liver-directed therapies including partial hepatectomy, ablation, or arterial chemo- and radioembolization may be useful. Resection is usually contraindicated in the presence of multifocal extrahepatic metastases, high-grade and poorly-differentiated carcinoma, liver disfunction, or diffuse bilobar involvement[55]. Previously, resection was only recommended if more than 90% of disease could be removed but more recent literature supports lowering this threshold to 70%[74-76]. Ablation is often reserved for the treatment of small metastases that do not qualify for surgical resection or may be done in addition to resection in the presence of multifocal disease. Arterial embolization, radioembolization, and chemoembolization can be used to diminish the secretory effects of functioning panNET. Liver transplantation is only considered in patients with significant hepatic tumor burden, without the presence of extrahepatic metastases, and is not routinely undertaken in metastatic panNET[1,55]. ^{68}Ga-DOTATATE PET/CT may be useful for determining suitability of patients for transplantation, as this technique allows for a whole-body acquisition in order to assess potential extrahepatic metastatic disease[35].

Symptom-directed therapy

Symptom-directed therapy plays an important role in the management of functioning panNETs. Treatment varies with each functioning panNET; common interventions include the use of diazoxide to suppress insulin secretion in insulinomas, and proton pump inhibitors to suppress hypersecretion by gastrinomas. Long-acting SSA may also be useful for controlling the secretory effects of these tumors, particularly vasoactive intestinal peptide-secreting tumors and glucagonomas[55].

CONCLUSION

Better understanding of the genetic and biological features of panNEN has led to significant changes in the diagnosis and management of these tumors. Imaging is crucial for diagnosing and staging of panNEN. CT and MR play a vital role in differentiating these tumors from other benign and malignant lesions of the pancreas. Recent studies indicate enhancement pattern of panNEN on cross sectional imaging and texture analysis may also be helpful in classifying these tumors or indicating prognosis. Diagnosis of panNEN is typically confirmed with EUS guided biopsy. Functional imaging techniques including SRS and PET/CT are very helpful in the management of panNEN. ^{68}Ga-DOTATATE and GLP-1R-based PET/CT may improve detection of occult lesions and their characterization. These techniques also have the potential to guide management, as information on somatostatin receptor expression and metabolic activity are useful for determining optimal treatment.

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MINIREVIEWS

Recent advances and new insights in the management of early-stage epidermal growth factor receptor-mutated non-small-cell lung cancer

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Abstract

Patients with early-stage non-small-cell lung cancer (NSCLC) are candidates for curative surgery; however, despite multiple advances in lung cancer management, recurrence rates remain high. Adjuvant chemotherapy has been demonstrated to significantly prolong overall survival (OS), but this benefit is modest

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and there is an urgent need for effective new therapies to provide a cure for more patients. The high efficacy of tyrosine kinase inhibitors (TKIs) against epidermal growth factor receptor-mutated (EGFR) in patients with advanced EGFR-mutated NSCLC has led to the evaluation of these agents in early stages of the disease. Multiple clinical trials have evaluated the safety and efficacy of EGFR TKIs as an adjuvant treatment, in patients with resected EGFR-mutated NSCLC, and shown that they significantly prolong disease-free survival (DFS), but this benefit does not translate to OS. Recently, an interim analysis of the ADAURA trial demonstrated that, surprisingly, osimertinib improved DFS. This led to the study being stopped early, leaving many unanswered questions about its potential effect on OS and its incorporation as a standard adjuvant treatment in this patient subgroup. These targeted agents are also being evaluated in locally-advanced disease, with promising results, although prospective studies with larger sample sizes are needed to confirm these results. In this article, we review the most relevant studies on the role of EGFR TKIs in the management of early-stage EGFR-mutated NSCLC.

Key Words: Non-small-cell lung cancer; Early stage; Epidermal growth factor receptor-mutated; Epidermal growth factor receptor-mutated-tyrosine kinase inhibitor; Adjuvant; Neoadjuvant

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Core Tip: Epidermal growth factor receptor-mutated (EGFR) tyrosine kinase inhibitors (TKIs) have changed the natural history of advanced EGFR-mutated non-small-cell lung cancer (NSCLC). Multiple clinical trials conducted in the adjuvant setting have shown that EGFR TKIs prolong disease-free survival (DFS) but not overall survival (OS). Osimertinib demonstrated a surprising improvement in DFS in an interim analysis of the ADAURA study, which led to the study being stopped early, and left many unanswered questions about its potential effect on OS. Locally-advanced disease is also an attractive situation for assessment of the efficacy of these agents, with encouraging results so far. We discuss the recent advances in the management of early-stage EGFR-mutated NSCLC.

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INTRODUCTION

Non-small-cell lung cancer (NSCLC) represents 85% of all lung cancers, and more than 50% of patients with NSCLC are diagnosed in advanced stages[1,2]. Only 25%-30% are diagnosed in early stages, making them candidates for curative surgical treatment[3-6]; however, more than 50% of these patients go on to have recurrence and die from the disease[3,6-9]. NSCLC has a high metastatic potential, even in early stages, and the aim of adjuvant treatment is to eradicate residual micrometastases[10]. Platinum-based adjuvant chemotherapy has been shown to prolong overall survival (OS), but with an absolute improvement in 5-year OS of only 4%[11,12]. Therefore, there is a need for new effective and minimally-toxic treatments to increase the cure rate.

Treatment with EGFR tyrosine kinase inhibitors (TKIs) in patients with metastatic EGFR-mutated NSCLC has been demonstrated to increase survival more than chemotherapy, changing the natural history of the disease in this subgroup of patients [13-17]. This has raised the question of whether a molecularly-targeted adjuvant treatment with EGFR TKIs could improve the modest benefit afforded by chemotherapy in patients with completely-resected EGFR-mutated NSCLC. Multiple EGFR

TKIs have been assessed in this setting and have shown a significant benefit in disease-free survival (DFS) but not OS[9,18-20].

Unresectable and potentially-resectable locally-advanced disease are also attractive settings for evaluating the role of these agents. Multiple phase II clinical trials have shown encouraging results[9,21-23], although many questions remain to be answered.

In this review, we discuss the most relevant studies evaluating the role of EGFR TKIs in resectable, potentially-resectable, and unresectable locally-advanced NSCLC with EGFR-activating mutation.

EGFR TKIS AS TREATMENT FOR RESECTABLE DISEASE

EGFR mutations are the most common oncogenic drivers in NSCLC, occurring in 10-15% of Caucasian patients[24-26] and approximately 30% of patients in Latin America [27], while in the Asian population the prevalence of EGFR mutations is significantly higher, around 50%[28,29].

The presence of EGFR-activating mutations in patients with NSCLC confers high sensitivity to treatment with EGFR TKIs. In phase III clinical trials, multiple EGFR TKIs have shown dramatic, long-lasting responses that have translated to longer survival[13-17,30-36], never before seen in patients with advanced NSCLC treated with chemotherapy, positioning these targeted agents as the standard treatment in patients with advanced EGFR-mutated NSCLC.

The prevalence of EGFR mutations in NSCLC, the results observed in advanced disease, and the clinical need for new treatments to help cure more patients in early stages have led to the evaluation of EGFR TKIs as adjuvant therapy (Table 1).

Initially, studies were carried out in a population that was not selected for the presence of EGFR mutations. Based on the rationale that high EGFR expression in NSCLC confers aggressiveness and poor response to chemotherapy, Goss *et al* [37] conducted the phase III trial BR19, which compared gefitinib for 2 yr *vs* placebo in 503 patients with resected stage IB-IIIA NSCLC, and found no differences in DFS or OS between the two arms. There were only 15 patients with EGFR mutations, and no benefit was observed for gefitinib in this small subgroup, either in DFS [hazard ratio (HR): 1.84, 95% confidence interval (CI): 0.44-7.73; $P = 0.395$], or OS (HR 3.16, 95%CI: 0.61-16.45; $P = 0.15$)[37]. Similarly, the phase III trial RADIANT evaluated erlotinib for 2 yr *vs* placebo, after completion of standard adjuvant treatment, in 973 patients with resected stage IB-IIIA NSCLC with EGFR expression/amplification. There were no significant differences in DFS or OS between the two arms. However, when the data from the 161 patients with EGFR-activating mutations were analysed, DFS was better with erlotinib (46.4 *vs* 28.5 mo; HR: 0.61, 95%CI: 0.384-0.981; $P = 0.039$), but this did not reach statistical significance due to the hierarchical analysis established in the study design. The 2-year DFS was 75% and 54% for erlotinib and placebo, respectively[38]. Although there was a marked difference between the two study arms in patients with EGFR mutation, it should be noted that certain imbalances in the patient characteristics may have influenced these results (more patients with stage IB in the erlotinib arm; in the placebo arm, more patients were in stage IIIA and 44% of patients did not receive previous adjuvant chemotherapy).

Following the discovery that the presence of EGFR mutation favours response to EGFR TKIs, multiple trials have been performed to evaluate these agents as adjuvant treatment in patients with EGFR mutations. SELECT was a phase II single-arm trial that included 100 patients with resected stage IA-IIIA EGFR-mutated NSCLC, who, after completing standard adjuvant treatment, received erlotinib for 2 years. The 2-year DFS was 88%, which was significantly higher than the 76% observed in historic controls ($P = 0.0047$). The 5-year DFS and OS were 56% and 86%, respectively. It is important to mention that of the 40 patients who had disease recurrence, this occurred during treatment in only 4; in the other 36 it occurred after stopping erlotinib[39]. In the phase III trial ADJUVANT/CTONG1104, 222 patients with resected stage II-IIIA EGFR-mutated NSCLC were randomly assigned to receive gefitinib for 2 yr or cisplatin plus vinorelbine for 4 cycles. With a median follow-up of 36.5 mo, the median DFS was significantly longer with gefitinib than with cisplatin plus vinorelbine (28.7 *vs* 18 mo; HR: 0.60, 95%CI: 0.42-0.87; $P = 0.0054$)[40]. However, with longer follow-up, no statistically significant difference was observed between the two arms for 3-year DFS (39.6% *vs* 32.5%; $P = 0.316$), 5-year DFS (22.6% *vs* 23.2%; $P = 0.928$), or OS (75.5 *vs* 62.8 mo; HR: 0.92, 95%CI: 0.62-1.36; $P = 0.674$)[41]. Likewise, Tada *et al* conducted the phase III IMPACT study, which included 234 patients with resected stage II-III EGFR-mutated NSCLC randomized to receive gefitinib for 2 yr or cisplatin plus vinorelbine

Table 1 Clinical trials of adjuvant epidermal growth factor receptor-mutated tyrosine kinase inhibitors in epidermal growth factor receptor-mutated non-small-cell lung cancer

Clinical trial	Type of trial	Sample size	Primary outcome	Stage	Treatment	Previous adjuvant chemotherapy	TKI Duration	DFS	OS
BR19, Goss *et al*[37]	Phase III	503 (15 with EGFR mutation)	OS	IB-IIIA	Gefitinib *vs* placebo	Yes (17% in gefitinib arm and 17% in placebo arm)	2 yr	HR 1.84; $P = 0.395$[1]	HR: 3.16; $P = 0.15$[1]
RADIANT, Kelly *et al*[38]	Phase III	973 (161 with EGFR mutation)	DFS (ITT population)	IB-IIIA	Erlotinib *vs* placebo	Yes (45.1% in erlotinib arm and 55.9% in placebo arm)[1]	2 yr	46.4 *vs* 28.5 mo; HR: 0.61; $P = 0.039$[1]	Median OS NR in both arms; HR: 1.09; $P < 0.001$[1]
SELECT, Pennell *et al*[39]	Phase II	100	2-yr DFS	IA-IIIA	Erlotinib	Yes (not reported)	2 yr	Mean DFS NR; 2-yr DFS 88%; 5-yr DFS 56%	Median OS NR, 5-yr OS 86%
ADJUVANT/CTONG1104, Zhong *et al*[40,41]	Phase III	222	DFS	II-IIIA	Gefitinib *vs* cisplatin-vinorelbine	No	2 yr	28.7 *vs* 18 mo; HR: 0.60; $P = 0.0054$ 3-yr DFS 39.6% *vs* 32.5%; $P = 0.316$ 5-yr DFS 22.6% *vs* 23.2%; $P = 0.928$	75.5 *vs* 62.8 mo; HR: 0.92; $P = 0.674$
IMPACT, Tada *et al*[42]	Phase III	234	DFS	II-III	Gefitinib *vs* cisplatin-vinorelbine	No	2 yr	36 *vs* 25.2 mo; HR: 0.92; $P = 0.63$	Median OS NR in both arms; HR: 1.03; $P = 0.89$
EVAN, Yue *et al*[43]	Phase II	102	2-yr DFS	IIIA	Erlotinib *vs* cisplatin-vinorelbine	No	2 yr	42.4 *vs* 21 mo; HR: 0.268; $P < 0.0001$ 2-yr DFS 81.4% *vs* 44.6%; $P = 0.0054$ 3-yr DFS 54.2% *vs* 19.8%; $P = 0.0460$	Median OS NR in both arms; HR: 0.165; $P = 0.0013$
Neal *et al*[44]	Phase II	46	2-yr DFS	IA-IIIA	Afatinib 3 mo *vs* 2 yr	Yes (52% in 3-mo arm and 45% in 2-yr arm)	3 mo *vs* 2 yr	42.8 *vs* 58.6 mo 2-yr DFS 81% *vs* 70%; $P = 0.55$	Median OS NR in both arms
ADAURA, Wu *et al*[45]	Phase III	682	DFS in stages II-IIIA	IB-IIIA	Osimertinib *vs* placebo	Yes (60% in both arms)	3 yr	Stages II-IIIA: NR *vs* 19.6 mo; HR: 0.17; $P < 0.001$ 2-yr DFS 90% *vs* 44%ITT: NR *vs* 27.5 mo; HR: 0.20; $P < 0.001$ 2-yr DFS 89% *vs* 52%	Median OS NR in both arms (immature OS data)

[1]Results in EGFR-mutated population. EGFR: Epidermal growth factor receptor; DFS: Disease-free survival; HR: Hazard ratio; ITT: Intention to treat; NR: Not reached; OS: Overall survival; TKI: Tyrosine kinase inhibitor.

for 4 cycles. The results were recently reported, with no differences observed in DFS (36 *vs* 25.2 mo; HR: 0.92, 95%CI: 0.67-1.28; $P = 0.63$) or OS (median not reached in either arm; HR: 1.03, 95%CI: 0.65-1.65; $P = 0.89$) between the two arms[42]. The ADJUVANT/CTONG1104 and IMPACT trials, with similar designs, showed an initial separation of the DFS curves, which overlap around 48 mo, suggesting that adjuvant treatment with EGFR TKIs only delays relapse.

The lack of results demonstrating a benefit in OS and the heterogeneous populations (stages IA-IIIA) included in the various clinical trials prompted the phase II

randomised trial EVAN, which evaluated erlotinib for 2 yr *vs* cisplatin plus vinorelbine for 4 cycles, as an adjuvant treatment, in a specific population of 102 patients with resected stage IIIA EGFR-mutated NSCLC who had received no previous treatment[43]. The median DFS was significantly longer with erlotinib than with chemotherapy (42.4 *vs* 21 mo; HR: 0.268, 95%CI: 0.136-0.531; $P < 0.0001$). Both 2-year DFS (81.4% *vs* 44.6%; $P = 0.0054$), and 3-year DFS (54.2% *vs* 19.8%; $P = 0.0460$) were significantly higher with erlotinib. However, this study had several limitations, including the small sample size and the high percentage of patients (35%) in the chemo-therapy arm who did not meet the per protocol population criteria (8 patients who did not receive chemotherapy and 11 major protocol deviations), which could have influenced the difference in DFS between the two study arms. Furthermore, in this study, PET scan was not performed as part of screening; this, in addition to the patients with stage IIIA having a high probability of micrometastatic disease[10], means that a percentage of patients, rather than an adjuvant treatment, could have been receiving treatment for advanced disease—a situation in which it is already known that EGFR TKIs are superior to chemotherapy. Afatinib, the second-generation EGFR TKI, which was the first to demonstrate a benefit in OS in patients with advanced EGFR-mutated NSCLC[13], was also assessed as an adjuvant, in a phase II clinical trial comparing afatinib for 2 yr *vs* afatinib for 3 mo, in 46 patients with resected stage IA-IIIA EGFR-mutated NSCLC, who had previously received standard adjuvant treatment. The 2-year DFS was numerically higher with 2 yr of afatinib than with 3 mo (81% *vs* 70%; $P = 0.55$), but this difference did not reach statistical significance, although it must be recognised that certain limitations of the study such as the small sample size and low percentage of patients who completed treatment in the 2-year group (41%) could have influenced the lack of statistical significance[44].

Osimertinib, a third-generation EGFR TKI, was evaluated as first-line treatment for EGFR-mutated NSCLC in the phase III trial FLAURA, showing longer survival and greater central nervous system (CNS) efficacy than erlotinib or gefitinib[15,16]. The high efficacy demonstrated by this agent in advanced disease and the lack of robust results supporting the use of EGFR TKIs as adjuvant treatment led Wu *et al* to conduct the phase III trial ADAURA. This included 682 patients with resected stage IB-IIIA EGFR-mutated NSCLC, who, after completing standard adjuvant chemotherapy, were randomly assigned to receive osimertinib or placebo for 3 yr. The primary outcome of the study was DFS in patients in stages II-IIIA. After an interim analysis that was not planned as part of the protocol, the independent monitoring committee recommended unblinding of the study, due to evidence of a clear benefit in favour of osimertinib. In patients with stage II-IIIA disease, osimertinib markedly improved DFS (not reached *vs* 19.6 mo; HR: 0.17, 99.06%CI: 0.11-0.26; $P < 0.001$) in comparison with placebo, the 2-year DFS being 90% and 44%, respectively. These results were consistent in the total population (median not reached *vs* 27.5 mo; 2-year DFS 89% *vs* 52%; HR: 0.20, 99.12%CI: 0.14-0.30; $P < 0.001$), and a reduction was also observed in risk of CNS recurrence or death (HR 0.18, 95%CI: 0.10-0.33)[45].

While the benefit observed with osimertinib in terms of DFS was striking, many questions were raised regarding whether these results, with a median follow-up of 22 mo in an adjuvant trial, were sufficient to position osimertinib as a standard treatment in this situation. Recently, Zhong *et al*[41] published the updated data from the ADJUVANT/CTONG1104 trial, confirming a benefit in DFS, but which ultimately did not translate to an OS benefit. In addition, multiple meta-analyses have analysed the role of EGFR TKIs as adjuvant treatment in patients with NSCLC with an EGFR-activating mutation, showing a benefit in DFS but not OS[46-48].

Although, overall, the trials with first- and second-generation TKIs showed a benefit in DFS, the high number of recurrences after stopping adjuvant treatment with EGFR TKIs in the different studies was striking. In the ADJUVANT/CTONG1104 trial, the difference in DFS observed between the two arms was smaller with a longer follow-up [41], while in the SELECT trial, 90% of recurrences occurred after stopping erlotinib [39]. These findings suggest that adjuvant treatment with EGFR TKIs delays recurrence but does not prevent it, and therefore does not appear to be able to change the natural history of the disease by curing more patients.

Although multiple studies have evaluated the role of EGFR TKIs as adjuvant therapy, the question of whether previous adjuvant chemotherapy is necessary remains unanswered. An indirect comparison of the DFS results from RADIANT, SELECT, and the phase II afatinib trial with those from the ADJUVANT/CTONG1104 trial suggests that giving an EGFR TKI after standard adjuvant treatment provides a greater benefit in DFS than giving an EGFR TKI as a sole adjuvant treatment[18]. In the ADAURA trial, 60% of patients in the osimertinib arm received adjuvant chemotherapy, which could have led to a greater benefit in the experimental arm, and the

lack of adjuvant chemotherapy in 40% of the control arm patients could have led to a more marked difference between the two arms.

It should be borne in mind that EGFR TKIs given for a prolonged period cause toxicity[38,39,44,45], which can negatively affect quality of life in patients who are considered disease-free. If we were to treat all patients with resected EGFR-mutated NSCLC with adjuvant EGFR TKIs, we would be over-treating a group of patients that may already be cured, meaning we would not be adding any benefit and only worsening their quality of life.

Another point under discussion is the response to EGFR TKIs in patients with recurrence after receiving adjuvant therapy. In the SELECT study, only one patient with recurrence during erlotinib treatment was found to have a T790M mutation, and 65% of patients with recurrence were retreated with erlotinib, reaching a median treatment duration of 13 mo[39]. Likewise, in the ADJUVANT/CTONG1104 trial, 36.8% of patients with recurrence in the gefitinib arm were treated with EGFR TKIs, achieving a response rate of 46.4%[41]. While the results available so far suggest that adjuvant treatment with EGFR TKIs does not appear to affect sensitivity to these agents in patients with recurrence, there is still insufficient evidence and we cannot draw definitive conclusions regarding the potential development of resistance to these agents.

Targeted treatments and immunotherapy have been shown to significantly prolong OS in advanced NSCLC; however, high prices make access difficult, so many patients cannot benefit from these agents. Osimertinib is a very expensive drug and the ADAURA study proposed a prolonged treatment, so it is reasonable to demand a strong benefit in OS that justifies its use, especially as there would be a group of patients receiving adjuvant osimertinib who may already be cured and would therefore be overtreated at the expense of toxicity and a very high economic cost[19,49,50].

Finally, a significant improvement in DFS that does not translate to a significant improvement in OS has been a constant finding in adjuvant studies of EGFR TKIs, which raises the issue of whether DFS is a suitable primary outcome in adjuvant studies[19]. Although, in the past, DFS was considered a surrogate for OS in NSCLC [51], this is only applicable for chemotherapy[50], and nowadays, in the era of targeted therapies, with more treatment options available, there is a greater probability that OS will be affected by subsequent treatments, as has been seen in multiple studies with EGFR TKIs in patients with advanced disease[52]. One possible explanation for the lack of OS benefit in adjuvant studies is that, in patients in the control arm, treatment with EGFR TKIs at the time of recurrence could have attenuated a potential benefit in OS, if present[20]. This makes us question whether we really should treat all these patients with adjuvant EGFR TKIs, if treating only patients with recurrence would achieve the same results. We must await the OS results from the ADAURA trial, but it is likely that these will be affected by the early termination of the study[19], and that we will never know if this dramatic benefit in DFS translates to a higher patient cure rate. Currently, the phase III trial ALCHEMIST (A081105) is underway, which compares erlotinib for 2 yr *vs* placebo, in patients with completely-resected stage IB-IIIA EGFR-mutated NSCLC, after standard adjuvant treatment. The primary outcome of this study is OS[53], and it could give us more information on the role of EGFR TKIs in this setting.

EGFR TKIS AS TREATMENT FOR POTENTIALLY-RESECTABLE LOCALLY-ADVANCED DISEASE

Locally-advanced NSCLC is associated with a poor prognosis[54]. Although such patients are treated with curative intent, the 5-year OS rates are low. In this situation, pathological complete response (pCR) after a preoperative treatment has been correlated with OS[55]. However, neoadjuvant chemotherapy achieves pCR rates that range from 0-16%[56]. Currently, ongoing research aims to translate the benefits from targeted therapy and immunotherapy to potentially-resectable disease.

Neodjuvant immunotherapy, with or without chemotherapy, is being assessed in multiple clinical trials, with promising results[57,58]. Provencio *et al* carried out the phase II clinical trial NADIM, in which a surprising pCR of 63% was reported with chemotherapy plus nivolumab[59].

EGFR TKIs are also being assessed for use as neoadjuvant treatment in NSCLC (Table 2). Zhong *et al*[41] carried out a small phase II trial in which they assessed the feasibility of giving neoadjuvant treatment guided by EGFR status, in 24 patients with stage IIIA NSCLC. Patients with mutated EGFR received erlotinib for 42 d, while

Table 2 Clinical trials of neoadjuvant epidermal growth factor receptor tyrosine kinase inhibitors in epidermal growth factor receptor-mutated non-small-cell lung cancer

Clinical trial	Study type	Sample size	Primary outcome	Stage	Treatment	TKI duration	RR	R0 resectability rate	PR	OS
Zhong *et al*[60]	Phase II	24 (12 with EGFR mutation)	RR	IIIA	Erlotinib (patients with EGFR mutation) *vs* carboplatin-gemcitabine (patients with native EGFR)	42 d	58.3% *vs* 25%; *P* = 0.18	50% *vs* 71.4; *P* = 0.59	16.7% *vs* 25%; *P* = 0.64	14.5 *vs* 28.1 mo; *P* = 0.201
Xiong *et al*[61]	Phase II	25	Resectability rate	IIIA	Erlotinib	56 d	42.1%	68.4%	50%	51.6 mo
Xiong *et al*[62]	Phase II	31 (15 with EGFR mutation)	Resectability rate	IIIA	Erlotinib *vs* cisplatin-based chemotherapy	4-7 wk	67% *vs* 19%	80% *vs* 50%	67% *vs* 38%	51 *vs* 20.9 mo
EMERGING-CTONG 1103, Zhong *et al*[63], Wu *et al*[64]	Phase II	72	RR	IIIA	Erlotinib *vs* cisplatin-gemcitabine	42 d (12 mo after surgery)	54.1% *vs* 34.3%; *P* = 0.092	73% *vs* 63%	MPR: 9.7% *vs* 0%	42.2 *vs* 36.9 mo; HR: 0.83; *P* = 0.513

EGFR: Epidermal growth factor receptor; HR: Hazard ratio; MPR: Major pathological response; OS: Overall survival; PR: Pathological response; RR: Response rate; TKI: Tyrosine kinase inhibitor.

patients with native EGFR received carboplatin plus gemcitabine for 3 cycles. Although the response rate (RR) was numerically higher with erlotinib (58.3% *vs* 25%; *P* = 0.18), there was no increase in the N2 pCR (16.7% *vs* 25%; *P* = 0.64) or in OS (14.5 *vs* 28.1 mo; *P* = 0.201)[60]. A different phase II single-arm trial included 25 patients with stage IIIA EGFR-mutated NSCLC treated with neoadjuvant erlotinib for 56 d, observing a RR of 42.1%, with a resectability rate of 68.4%. On pathology, 50% partial responses were reported, but no complete response[61]. Similarly, Xiong *et al* conducted a phase II clinical trial in patients with stage IIIA NSCLC, in which they compared neoadjuvant treatment with erlotinib for 4-7 wk (15 patients with EGFR mutation) and cisplatin-based doublet chemotherapy for 2 cycles (16 patients without EGFR mutation), observing a RR (67% *vs* 19%) and an OS (51 *vs* 20.9 mo) that were numerically higher in the patients treated with erlotinib. The pathological response was higher in the erlotinib group (67% *vs* 38%), although this difference was not statistically significant and there was no pCR in this group[62]. Finally, the phase II trial EMERGING-CTONG 1103 included 72 patients with stage IIIA EGFR-mutated NSCLC, randomly assigned to erlotinib (42 d neoadjuvant and 12 mo adjuvant) *vs* cisplatin plus gemcitabine (2 cycles neoadjuvant and 2 cycles adjuvant). There were no significant differences in RR (54.1% *vs* 34.3%; *P* = 0.092) or OS (45.8 *vs* 39.2 mo; HR: 0.77, 95%CI: 0.41-1.45; *P* = 0.417), and pCR was not observed in either arm[63]. Final analysis of OS was recently reported, with similar results (42.2 *vs* 36.9 mo; HR: 0.83, 95%CI: 0.47-1.47; *P* = 0.513)[64].

The small sample sizes and heterogeneity of these phase II trials do not allow us to draw definitive conclusions regarding efficacy. There was a remarkable lack of pCR in these studies; in addition, the response rates appear to be lower than those observed with EGFR TKIs as first-line treatment[31,33,34,36], which could be due to the short preoperative treatment duration in these trials. Although neoadjuvant treatment with these agents appears feasible, there remain many unanswered questions, such as the risk of disease flare after stopping EGFR TKI treatment[65]; randomised trials with larger sample sizes are needed to provide more data on the safety and efficacy of these agents in potentially-resectable disease. Currently underway is the phase III clinical trial NeoADAURA (ClinicalTrials.gov number, NCT04351555), which compares osimertinib for 9 wk with or without chemotherapy for 3 cycles *vs* chemotherapy alone for 3 cycles, as neoadjuvant treatment, in patients with stage II-IIIB EGFR-mutated NSCLC, followed by adjuvant osimertinib for 3 yr. This trial could provide more information on the optimal duration of preoperative treatment, the role of chemotherapy in this scenario, and the need for adjuvant treatment.

Table 3 Clinical trials of pidermal growth factor receptor tyrosine kinase inhibitors in the management of unresectable pidermal growth factor receptor-mutated non-small-cell lung cancer

Clinical trial	Type of study	Sample size	Primary outcome	Stage	Treatment	TKI duration	RR	PFS	OS
RECEL, Xing *et al*[70]	Phase II	40	PFS	III unresectable	Erlotinib + RT *vs* cisplatin-etoposide + RT	2 yr	70% *vs* 61.9%; $P = 0.744$	24.5 *vs* 9 mo; HR: 0.104; $P < 0.001$	Not reported
Lee *et al*[71]	Phase II	59 (12 with EGFR mutation)	RR, toxicity and OS	III unresectable	EGFR mutation: erlotinib x 3 → erlotinib+RT → erlotinib x 6 *vs* erlotinib x 3 → cisplatin-irinotecan+RT Native/unknown EGFR: cisplatin-irinotecan × 3 → cisplatin-irinotecan+RT *vs* cisplatin-irinotecan+RT → cisplatin-irinotecan x 3	33 wk	EGFR mutation: 71.4% *vs* 80% Native/unknown EGFR: 70% *vs* 73.9%	EGFR mutation: 11.6 *vs* 8.1 mo Native/unknown EGFR: 9 *vs* 12.3 mo	EGFR mutation: 39.3 *vs* 31.2 mo Native/unknown EGFR: 16.3 *vs* 25.3 mo Mutated *vs* native EGFR: 74.8 *vs* 25.3 mo, $P = 0.034$
LOGIK0902/OLCSG0905, Saeki *et al*[73]	Phase II	20	2-yr OS	III unresectable	Gefitinib cisplatin-docetaxel+RT	8 wk	85%	2-yr PFS 36.9%	2-yr OS 90%

HR: Hazard ratio; OS: Overall survival; PFS: Progression-free survival; RR: Response rate; RT: Radiotherapy; TKI: Tyrosine kinase inhibitors.

EGFR TKIS AS TREATMENT FOR UNRESECTABLE LOCALLY-ADVANCED DISEASE

The phase III PACIFIC trial demonstrated that maintenance with durvalumab after chemoradiotherapy, in patients with unresectable stage III NSCLC, significantly prolonged DFS and OS[66,67], positioning it as the standard treatment for these patients. Despite the good outcomes with this treatment strategy, approximately 44% of patients had progression and died from the disease[67]. Therefore, there is a need for new biomarker-guided therapies that would allow us to appropriately select the best treatment for each patient.

The PACIFIC trial subgroup analysis suggests that patients with EGFR mutation may benefit less from chemoradiotherapy followed by durvalumab[66]. This is probably due to the biology of EGFR-mutated NSCLC, which is associated with a higher risk of metastasis, meaning these patients obtain a greater benefit from local treatment such as chemoradiotherapy[68,69]. Thus, the optimal treatment in this patient subgroup is unknown.

Preclinical studies suggest that EGFR TKIs have a radiosensitizing effect[21,22]. This has prompted several clinical trials to evaluate the role of these targeted agents in unresectable locally-advanced disease (Table 3). The phase II trial RECEL compared erlotinib (for 2 yr) or cisplatin plus etoposide, concomitantly with radiotherapy in 40 patients with unresectable stage III EGFR-mutated NSCLC, and demonstrated that erlotinib plus radiotherapy significantly prolonged DFS (24.5 *vs* 9 mo; HR: 0.104, 95%CI: 0.028-0.389; $P < 0.001$) compared to chemoradiotherapy[70]. A different phase II study by Lee *et al* included 59 patients with unresectable stage III NSCLC, of whom

12 had an EGFR-activating mutation. Patients with mutated EGFR were randomised to erlotinib for 3 cycles, followed by erlotinib plus radiotherapy, followed by erlotinib for 6 cycles, *vs* erlotinib for 3 cycles followed by chemoradiotherapy with cisplatin plus irinotecan; patients with native/unknown EGFR status were randomised to cisplatin plus irinotecan for 3 cycles before or after chemoradiotherapy with cisplatin plus irinotecan. Patients with mutated EGFR had a significantly longer OS (74.8 *vs* 25.3 mo, $P = 0.034$) than patients with native EGFR[71]. Gefitinib has also been assessed, in the phase II trial LOGIK0902/OLCSG0905[72], which included 20 patients with unresectable stage III EGFR-mutated NSCLC who were treated with gefitinib for 8 wk followed by chemoradiotherapy with cisplatin plus docetaxel, and found a RR of 85%, a 2-year DFS rate of 36.9%, and a 2-year OS of 90%[73].

Although these phase II studies show encouraging results, they must be confirmed in phase III clinical trials. Currently, the phase III LAURA trial is underway, comparing osimertinib until progression *vs* placebo, as maintenance treatment after standard chemoradiotherapy[74].

CONCLUSION

Adjuvant treatment with first- and second-generation EGFR TKIs, in patients with resected EGFR-mutated NSCLC, has demonstrated a benefit in DFS, which does not translate to OS. Surprisingly, in the ADAURA trial, the third-generation EGFR TKI osimertinib prolonged DFS in these patients; however, certain limitations of the design of this study and its early termination based on a benefit in DFS only, raise questions about its use as a standard adjuvant treatment. The OS data from the ADAURA trial and the results of the ALCHEMIST trial will confirm if EGFR TKIs have a role as adjuvant treatment.

These targeted therapies are also undergoing evaluation in potentially-resectable and unresectable locally-advanced disease, with encouraging results; however, we must await the results of the phase III trials NeoADAURA and LAURA, which should provide more data on the safety and efficacy of EGFR TKIs in these situations.

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ORIGINAL ARTICLE

Retrospective Study

A biomarker study in Peruvian males with breast cancer

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Abstract

BACKGROUND

Breast cancer (BC) frequency in males is extremely low and tumor features vary from its female counterpart. Breast cancer clinical and pathological features differ by race in women. Tumor infiltrating lymphocyte (TIL) levels, mismatch repair (MMR) protein loss, androgen receptor (AR) expression, and PIK3CA gene mutations are predictive biomarkers of response to biological therapy in female BC. There is limited information about clinical and pathological features as well as predictive biomarkers in males of non-Caucasian races with BC.

AIM

To investigate clinicopathological features and biomarkers of BC tumors in males and their prognostic value in Peruvian population.

METHODS

This study looked at a single-institution series of 54 Peruvian males with invasive BC who were diagnosed from Jan 2004 to June 2018. Standard pathological features, TIL levels, MMR proteins, AR immunohistochemistry staining, and PIK3CA gene mutations were prospectively evaluated in cases with available

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Institutional review board statement: This study was reviewed and approved by the Instituto Nacional de Enfermedades Neoplasicas Institutional Review Board. Personal and filiation data including identity of every patient was protected with an added code in the excel table. It is a retrospective case series that does not have any not activity or contact with the patients.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

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paraffin material. Percentage of AR and estrogen receptor (ER) positive cells was additionally calculated by software after slide scanning. Statistical analyses included association tests, intraclass correlation test and Kaplan Meier overall survival curves.

RESULTS

The median age was 63 years and most cases were ER-positive (85.7%), HER2 negative (87.2%), Luminal-A phenotype (60%) and clinical stage II (41.5%) among our male breast tumors. Median TIL was 10% and higher levels tended to be associated with Luminal-B phenotype and higher grade. AR-positive was found in 85.3% and was correlated with ER (intraclass index of 0.835, $P < 0.001$). Loss of MMR proteins was found in 15.4% and PIK3CA mutation (H1047R) in 14.3% (belonged to the Luminal-A phenotype). Loss of MMR proteins was associated with AR-negative ($P = 0.018$) but not with ER ($P = 0.43$) or TIL ($P = 0.84$). Early stages ($P < 0.001$) and lower grade ($P = 0.006$) were associated with longer overall survival. ER status, phenotype, AR status, TIL level, MMR protein loss nor PIK3CA mutation was not associated with survival ($P > 0.05$).

CONCLUSION

Male BC is usually ER and AR positive, and Luminal-A. MMR loss and PIK3CA mutations are infrequent. Stage and grade predicted overall survival in our South American country population.

Key Words: Male breast neoplasm; Androgen receptor; Tumor-infiltrating lymphocyte; Mismatch repair protein; PIK3CA mutation

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Core Tip: Most male breast cancers were estrogen receptor-positive, HER2-negative, androgen receptor (AR)-positive, and Luminal A phenotype. Loss of mismatch repair (MMR) and PIK3CA mutations was found in around 15% of the cases. AR was correlated with ER expression and without loss of MMR proteins. Stage and grade are prognostic features in Peruvian male breast cancer.

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INTRODUCTION

Male breast cancer (BC) represents less than 1% of mammary carcinomas. Male BC has a higher expression rate of estrogen receptor (ER), higher proliferative activity, and generally more aggressive behavior than in females. The etiological attribution of inheritance for BC is more prevalent among males than females, but only a small fraction is attributed to BRCA2 mutation, and even less to BRCA1[1-5].

Combination of immunohistochemical biomarkers predicts response to therapy and allows us to classify molecular subtypes in female BC[6]. Molecular subtypes distribution and clinical features differ regarding racial populations, for instance, high triple negative breast cancer (TNBC) prevalence and young age at diagnosis are described in Latin-American women[6]. However, only a few small reports have evaluated frequency of molecular BC subtypes in Latin-American males [7,8].

Androgen-receptor (AR) expression[9], tumor-infiltrating-lymphocytes (TIL)[10], loss of mismatch-repair (MMR) proteins (biomarker of the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome)[11] and PI3K mutations[12] have been associated with response to target therapy in female BC. There is a need to develop epidemiological and therapeutic information in the male counterpart. This work aims

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to evaluate the prevalence of clinical and molecular biomarkers of BC tumors in Peruvian males, as well as their impact on survival.

MATERIALS AND METHODS

Study design and Patients

Fifty-four male BC cases who were histologically diagnosed at the Instituto Nacional de Enfermedades Neoplasicas between 2004 and 2018 were included. Clinical information was obtained from the patients' medical records. Live status of patients with not accurate follow-up was obtained from the Peruvian national registry (https://www.reniec.gob.pe) through the Epidemiology Department of the institution. The institutional review board waived informed consent and approved this retrospective case series.

Routine pathological examination

TIL assessment on Hematoxylin and Eosin-stained slide was possible in 42 available samples. TIL and histological grade was prospectively evaluated by three experienced pathologists (JS, ET and HG), who were blinded to the clinical data, following international recommendations[7].

Immunohistochemistry staining

Tissue samples were fixed in 10% buffered formalin to obtain 4 μm paraffinized histological sections from 35 available tissue paraffin blocks. Sections were transferred onto adhesive slides and were dried at 60 ℃ for 30 min. After incubation with the primary antibodies, immunodetection was performed using biotinylated anti-mouse immunoglobulin, followed by peroxidase-labeled streptavidin. The labeled streptavidin biotin kit was used, and 3,3'-diaminobenzidine chromogen was used as a substrate. Immunohistochemical staining for androgen-receptor protein (AR antibody, Dako), estrogen-receptor protein (ER antibody, Zhongshan Bio), Mut L Protein Homolog protein (MLH1 antibody, Dako), DNA mismatch repair protein Msh2 (MSH2 antibody, Dako), MutS Protein Homolog 6 protein (MSH6, Dako) and Postmeiotic Segregation Increased 2 protein (PMS2 antibody, Dako) were carried out according to the manufacturer's instructions. Normal prostatic tissue was used as a positive control of AR. Phosphate-buffered saline was used to replace the primary antibody and served as the negative control.

Tumors that had more than 10% of cells exhibiting a moderate or strong intensity of AR expression were considered positive[7]. Additionally, slides of AR and ER staining were scanned in BX63 Olympus (Tokyo, Japan) and the analysis was performed through Visiopharm software in 34 male BC cases. Negative and positive cells were marked in blue and green by TissueMorph Software, (Visopharm, Hoerlson, Denmark), and the proportion of cell count was obtained through the ratio of AR and ER on number of positive overall cells in 5 high power fields (Figure 1) under supervision of a pathologist as previously described (JS)[7]. Non-malignant stromal cells were used as internal positive controls for MLH1, MSH2, MSH6, and PMS2, while the loss of MMR was considered when lacking nuclear staining for at least one was found[13].

Determination of PIK3CA mutation

Tumor DNA from paraffin-embedded samples was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen; Hilden, Germany) in the available 14 tumor samples. TaqMan-based real-time PCR analysis was conducted using a LightCycler® 96 Real-Time PCR System (Roche Applied Science, Mannheim, Germany) to detect the three ''hot spot'' PIK3CA mutations (H1047R, E545K and E542K). Custom TaqMan primers and probes were designed for the PIK3CA mutations (PI3KCA 760: c.1624 G (VIC) >A (FAM), PI3KCA 763: c.1633 G (VIC) >A (FAM), PI3KCA 775: c.3140 A (VIC) >G (FAM), ThermoFisher scientific). The thermal cycler protocol was as follows: 10 min at 96 C, 39 cycles at 60 ℃ for 2 min, 98 ℃ for 30 s, and 60 ℃ for 1 min. All samples were analyzed in a single assay for each mutation. The mutational threshold was determined by measuring the WT HDx FFPE reference standards (Horizon Diagnostics, Cambridge, UK), and was 1%. In contrast, the threshold of reagents inducing false positives was assumed to be 0.5% of the mutation frequency.

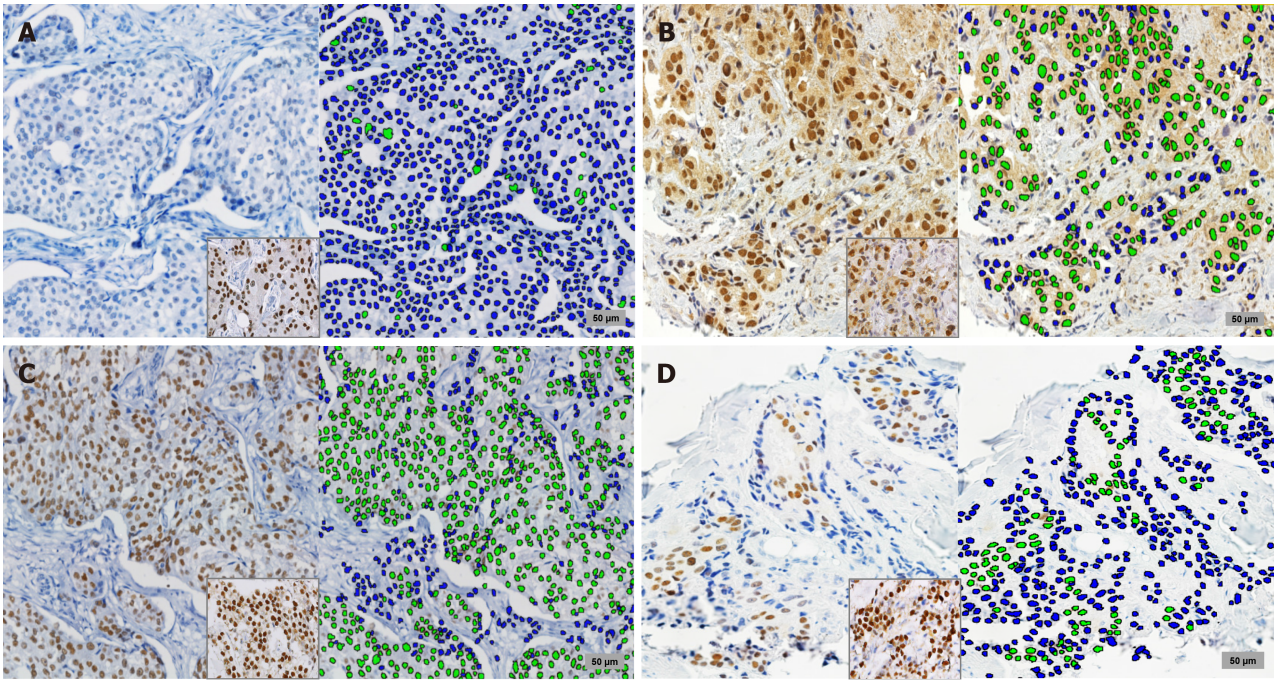


Figure 1 Immunohistochemical staining and automated scoring. Sections show positive staining for androgen receptor (A and B) and estrogen receptor (C and D) from the same tissue areas and the positive control. In the left, sections show images of the immunohistochemical studies (× 20). In the right, the automated analysis is presented, where negative nuclei are highlighted in blue and positive nuclei in green.

Statistical analyses

Associations of clinical-pathological variables were performed by the Chi-square test or the Fisher's exact test. Associations of ordinary variables were performed by the Kruskal-Wallis test. Identification of co-expression of AR and ER was performed by the intraclass correlation test. Overall survival (OS) analysis was estimated using the Kaplan–Meier method. Log-rank or Breslow tests were used to find differences between categories, according to the case. A $P < 0.05$ was considered significant. Analyses were performed using the SPSS statistical package (version 26.0.0, IBM SPSS Statistical).

RESULTS

Patient's characteristics

There were included 54 male BC patients and their median age was 63 years. Twenty-eight (51.9%) tumors were located on the left side, concurrent in-situ component in 41.4% and the most frequent clinical stage was II (41.5%) (Table 1). A family cancer history was found in 12 (22.2%) cases. Regarding treatment, mastectomy, tumorectomy and no primary resection were performed in 72.2%, 7.4% and 20.4%, respectively. Axillary dissection, sentinel node biopsy and no study of nodes were performed in 59.3%, 5.5% and 35.2%, respectively. Neoadjuvant and adjuvant chemotherapy was administrated in 22.2 and 35.2% of the resected cases, while adjuvant radiation was used in 44.4%. Tamoxifen was used in ER-positive cases. Recurrence was found in 28.9% (11/38) of the cases who underwent a curative surgical treatment.

Biomarkers, clinical-pathological features, and the correlations

Most cases were ER-positive (85.7%), HER2-negative (87.2%) and belonged to Luminal-A phenotype (Table 1). ER status was not associated with MMR-loss (P = 0.43). No association between ER percentage and MMR-loss was found (P = 0.22).

Positive AR status was found in 85.3% of patients and the two TNBC cases were negative for AR. MMR-loss was associated with AR-negative (75% *vs* 10%, P = 0.018). A lower median percentage of AR-positive cells was found in cases with MMR-loss (5% *vs* 70%, P = 0.02).

Evaluation of similar areas for ER and AR found that a median of 3738 cells (52.9%) (range 0%-87%) from 7577 cells (range 2139-11883 cells) were positive for ER, and 3795

Table 1 Clinical-pathological features and overall survival impact in male breast cancer

Features	*n*	%	OS-5yr	*P* value
Age				
Median (yr)	63			0.105[a]
< 63	25	53.7	56.0	
≥ 63	29	46.3	69.0	
Clinical stage (*n* = 53)				< 0.001[a]
I	2	3.8	100.0	
II	22	41.5	86.4	
III	20	37.7	50.0	
IV	9	17.0	22.2	
Histological grade (*n* = 46)				0.006[a]
1	7	15.2	85.7	
2	26	56.5	73.1	
3	13	28.3	30.8	
sTIL (*n* = 42)				0.397[b]
< 10%	12	28.6	50.0	
≥ 10%	30	71.4	66.7	
ER status (*n* = 49)				0.567[a]
Positive	42	85.7	66.7	
Negative	7	14.3	57.1	
PgR status (*n* = 49)				0.305[a]
Positive	43	87.8	67.4	
Negative	6	12.2	50.0	
HER2 status (*n* = 47)				0.088[b]
Positive	6	12.8	33.3	
Negative	41	87.2	70.7	
Ki67 (*n* = 40) (median 5%)				0.232[a]
0%-5%	22	55.0	59.1	
> 5%	18	45.0	66.7	
Phenotype (*n* = 45)				0.356[a]
Luminal A	27	60.0	63.0	
Luminal B	13	28.9	76.9	
TNBC	5	11.1	40.0	
AR (*n* = 35)				0.294[a]
0%-9%	5	14.3	60.0	
≥ 10%	30	85.7	63.3	
Mismatch repair loss (*n* = 26)				0.501[a]
No	20	76.9	65.00	
Yes	4	15.4	75.00	
Undetermined	2	7.7	100.00	
PIK3CA mutation H1047R (*n* = 14)				0.844[b]
Positive	2	14.3	50.0	

Negative	12	85.7	75.0

[a]*P*: Breslow.
[b]*P*: Log Rank. OS: Overall survival; TIL: Tumor infiltrating lymphocyte; TNBC: Triple negative breast cancer.

cells (55%) (range 0%-87.8%) from 7366 (range 2520-12135 cells) were positive for AR. Intraclass correlation coefficient of the means for AR and ER was 0.835 ($P < 0.001$) (Figure 2). A lower median of AR-positive tumor cell count proportion was also found in cases with MMR-loss (8% *vs* 65%, $P = 0.018$). No association between median of ER-positive tumor cell count proportion and MMR-loss was found ($P = 0.163$).

Median TIL was 10% and higher levels tended to be associated with Luminal-B ($P = 0.058$) (Figure 2). TIL ≥ 50% was associated with higher grade ($P = 0.039$), but not to age ($P = 0.44$), clinical stage ($P = 0.59$), MMR-loss (20% *vs* 15%, $P = 0.72$) nor PIK3CA mutation status ($P = 0.53$).

Loss of MMR protein expression was found in 4 (15.4%) cases. It was not associated with age ($P = 0.69$), clinical-stage ($P = 0.68$), grade ($P = 0.53$), molecular subtypes ($P = 0.91$) or PIK3CA mutation status ($P = 0.41$).

PIK3CA mutations were found in 2 (14.3%) of the 14 evaluated cases and both were positive for H1047R (1321 copies/microL-15.07% and 1019 copies/microL-18.06%). Both cases belonged to the Luminal-A phenotype.

The association of prognostics and clinical-pathological features

Longer OS was associated with early stage ($P < 0.001$) and lower grade ($P = 0.006$). Survival was not associated with ER status ($P = 0.305$), phenotype ($P = 0.152$), AR status ($P = 0.613$), TIL level ($P = 0.397$), MMR protein loss ($P = 0.501$) nor PIK3CA mutation status ($P = 0.844$).

DISCUSSION

We found a high frequency of AR expression and low frequency of both MMR-loss and PIK3CA mutations in our BC series in male Peruvian population.

Expression of AR was found in 85% of our male BC cases which is in the previously described range (40%–90%)[1], however we didn't find its association with prognosis. Androgen-receptor is a key driver of proliferation and cell survival, and although some retrospective male BC series describe its association with better prognosis[3] others like ours not[4]. Additionally, recent basic research and clinical trials describe that AR can predict activity of targeting drugs[9].

Our findings of high rates of ER-positive status and Luminal phenotype have been extensively described in Caucasian series[2,5]. Large sample size studies describe that ER-positive status would have a favorable prognostic effect (similar to the female BC) and predicts response to endocrine modulation[5,14,15]. The fact that we did not find an association with OS could be because of the small size of our series.

There was absence of HER2 enriched phenotype in our series and HER2- positive status was very infrequent. This corroborates previously published information that HER2 overexpression is less frequent than in female series, but it is expected to behave as a predictive feature to anti-HER2 therapies[2,5].

Our findings of 15% of cases with MMR loss have not previously been described in male BC. The HNPCC syndrome is an autosomal dominant genetic disorder characterized by predisposition to extracolonic malignancies at various sites including BC, is detected by loss of MMR proteins and is associated with high response to anti-PDL1 therapy. Studies with small sample size in female BC describe the lack or reduced expression of hMSH2 and hMLH1 in less than 20%[11,16]. Boyd *et al*[17] described a case of male BC belonging to a large HNPCC kindred that harbors a germline mutation of the MLH1. And recently, Piscuoglio *et al*[2] developed a pathway and network analysis of 241 genes in a series of 59 male BC and described an enrichment of mutations affecting DNA repair-related genes. We found that MMR-loss was associated with the previously described marker of endocrine response[3,4], the lower AR expression. Furthermore, this association appears to be specific for this steroid marker and not for ER, despite AR and ER expression were co-related. Haricharan *et al*[18] suggest that alteration in DNA damage repair genes could produce endocrine resistance because of the finding that defects in MMR pathway genes doesn't allow an accurate CDK4 suppression by endocrine therapy.

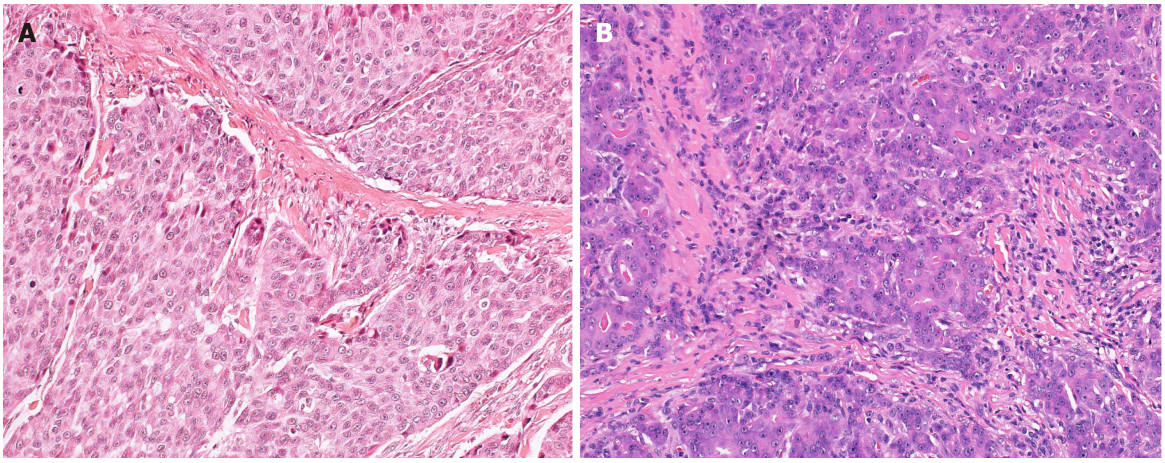


Figure 2 Evaluation of tumor infiltrating lymphocytes on hematoxylin-eosin staining (× 20). A: A Luminal-A breast cancer with low TIL level; B: A Luminal-B breast cancer with high TIL level.

PIK3CA mutations were found in only 2 of 14 evaluated male BC cases that is lower than previously reported by our group in women with BC[19,20]. Piscuoglio *et al*[2] sequenced 241 genes in 59 male BC and found that most recurrent mutations affected the PIK3CA gene (20%). However, these rates would be lower than their female counterparts that have been detected in approximately 30%-40% of female BC[21]. In vitro and translational research in tumor samples from clinical trials found that PIK3CA mutations reduce sensitivity to anti-HER2 drugs[22] and endocrine modulation[19,20]. Additionally, a recent study demonstrates that Alpelisib, a drug targeting the PIK3CA pathway, has activity in breast cancer cases with presence of the mutation [12].

TIL is a well-described prognostic and predictive biomarker for anti-PDL1-therapy in female BC[10]. Vermeulen *et al*[22] found that high TIL density evaluated by a method different than the currently recommended in international guidelines was associated with Luminal-B HER2-positive subtype and longer OS in a 1483 male BC series. We also found a trend of higher TILs in Luminal-B subtype but not a relationship with longer OS (P = 0.378). Contrary to the female counterpart or other malignancies, we did not find that higher TIL levels were associated with AR or ER percentage nor with MMR-loss.

Finally, our finding that early stage and lower grade features achieve significant association with better prognosis is consistent with previous reports, and supports the current management of this entity[2,5].

The weakness of our study was that several patients were lost of follow-up; however, obtaining information about their live status from the national registry allowed us to build OS curves. The number of included cases is also small because it is a very unusual entity, representing less than 0.5% of their female counterpart (18552 female new BC cases diagnosed in our center during the same period). Our strongness is the prospective evaluation of biomarkers by pathologists and biologist authors. Our results serve to complement male BC knowledge in the South American population, which has been under-studied.

CONCLUSION

We conclude that early-stage and low-grade features identify favorable prognoses in male BC. Most Peruvian BC cases are ER-positive, HER2-negative, AR-positive and Luminal-A tumors. MMR loss and PIK3CA mutations are infrequent, and MMR loss was associated with AR negative.

ARTICLE HIGHLIGHTS

Research background

Information about clinicopathological features associated with treatment response and

prognosis has been extensively described for female breast cancer, and differences regarding races has been described. Breast cancer in males is much less frequent, has important clinicopathological differences and is less studied than their female breast cancer counterpart.

Research motivation

Discussion and new information about features and biomarkers of Breast cancer (BC) in males have been included in recent cancer-related meetings, and more than 30000 articles have been published in the last two years. However, very few of them have evaluated a South American population.

Research objectives

To describe rates of currently accepted biomarkers for prognosis and for prediction of treatment response in Peruvian males with BC.

Research methods

Clinical files and tumor slides were reviewed. Tumor-infiltrating lymphocytes, mismatch repair proteins (MMR), PIK3CA gene mutations, estrogen (ER) and, androgen receptors (AR) were prospectively evaluated in available paraffin material.

Research results

In our series of 54 Peruvian males with invasive breast cancer, we found that most cases were Luminal-A phenotype (60%), ER-positive (85.7%), AR-positive (85.3%), and the median of tumor-infiltrating lymphocytes was 10%. MMR loss was found in 15.4% and PIK3CA mutation (H1047R) in 14.3% (all in the Luminal-A group). MMR loss was associated with AR-negative (P = 0.018). Longer overall survival was associated with early stages (P < 0.001) and lower grade (P = 0.006).

Research conclusions

Most breast cancer tumors in Peruvian males are ER and AR-positive, and MMR loss and PIK3CA mutations are infrequent. MMR loss was associated with hormone receptor-negative.

Research perspectives

Biomarkers identified for women with breast cancer need to be validated for the male counterpart. Research in race disparities needs to be extended also for males of non-Caucasian races. Association between MMR loss and activity of AR pathways requires further evaluation.

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ORIGINAL ARTICLE

Retrospective Study

Jejunostomy in the palliative treatment of gastric cancer: A clinical prognostic score

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Abstract

BACKGROUND

Clinical stage IV gastric cancer (GC) may need palliative procedures in the presence of symptoms such as obstruction. When palliative resection is not possible, jejunostomy is one of the options. However, the limited survival of these patients raises doubts about who benefits from this procedure.

AIM

To create a prognostic score based on clinical variables for 90-d mortality for GC patients after palliative jejunostomy.

METHODS

We performed a retrospective analysis of Stage IV GC who underwent jejunostomy. Eleven preoperative clinical variables were selected to define the score categories, with 90-d mortality as the main outcome. After randomization, patients were divided equally into two groups: Development (J1) and validation (J2). The following variables were used: Age, sex, body mass index (BMI), American Society of Anesthesiologists classification (ASA), Charlson Comorbidity index (CCI), hemoglobin levels, albumin levels, neutrophil-lymphocyte ratio (NLR), tumor size, presence of ascites by computed tomography (CT), and the number of disease sites. The score performance metric was determined by the area under the receiver operating characteristic (ROC) curve (AUC) to define low and high-risk groups.

RESULTS

Of the 363 patients with clinical stage IVCG, 80 (22%) patients underwent

conflicts of interest that might be relevant to the contents of this manuscript.

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jejunostomy. Patients were predominantly male (62.5%) with a mean age of 62.4 years old. After randomization, the binary logistic regression analysis was performed and points were assigned to the clinical variables to build the score. The high NLR had the highest value. The ROC curve derived from these pooled parameters had an AUC of 0.712 (95%CI: 0.537–0.887, P = 0.022) to define risk groups. In the validation cohort, the diagnostic accuracy for 90-d mortality based on the score had an AUC of 0.756, (95%CI: 0.598–0.915, P = 0.006). According to the cutoff, in the validation cohort BMI less than 18.5 kg/m^2 (P < 0.001), CCI ≥ 1 (P = 0.001), ASA III/IV (P = 0.002), high NLR (P = 0.012), and the presence of ascites on CT exam (P = 0.004) were significantly associated with the high-risk group. The risk groups showed a significant association with first-line (P = 0.012), second-line chemotherapy (P = 0.009), 30-d (P = 0.013), and 90-d mortality (P < 0.001).

CONCLUSION

The scoring system developed with 11 variables related to patient's performance status and medical condition was able to distinguish patients undergoing jejunostomy with high risk of 90 d mortality.

Key Words: Stomach neoplasms; Gastric cancer; Palliative surgery; Jejunostomy; Gastric cancer with outlet obstruction; Stage IV gastric cancer

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Core Tip: This is a retrospective study to evaluate the outcomes of jejunostomy in clinical stage IV gastric cancer patients, and create a scoring system based on clinical variables to identify the best candidates for this approach and avoid futile procedures. We analyzed 80 patients divided into a development and validation cohort. The score had an accuracy of 75.6% in the validation cohort, and was able to properly identify the cases with high risk of 90-d mortality.

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INTRODUCTION

Gastric cancer (GC) represents the third leading cause of cancer mortality worldwide [1]. Surgical treatment remains the standard of care for patients with resectable GC. Nevertheless, many patients at the time of diagnosis have already locally unresectable tumors or signs of systemic disease (clinical stage IV GC patients). As a result, palliative procedures are indicated in the presence of symptoms, including bleeding, perforation, or obstruction.

Palliative gastrectomy is the preferred treatment option for locally advanced GC with gastric outlet obstruction whenever it is feasible. However, if the tumor cannot be resected, the placements of endoscopic stents or gastrojejunostomy are additional options for distal lesions, according to the patient's life expectancy[2,3]. Unfortunately, some tumors may be too large or located in the upper third of the stomach, not allowing performing the gastrojejunostomy proximal to the tumor. In this scenario, a nasoenteric tube or jejunostomy are the main alternatives. Although jejunostomy enables the use of enteral nutrition, the patient persists with the obstruction and does not tolerate a full oral diet, with negative impact on quality of life.

Most studies evaluate the outcomes concerning placing a feeding jejunostomy at the time of gastroesophageal resection[4,5]. There are few reports concerning the influence of clinical and treatment variables on the outcome of palliative jejunostomy. Questions about improving survival and quality of life, in contrast to the morbidity and mortality rates in these cases, remain unanswered. Consequently, there are still doubts about

who benefits from jejunostomy.

Thus, the aim of this study was to analyze the clinical characteristics and prognosis of stage IV patients who underwent palliative jejunostomy due to obstructive GC, and create a prognostic score for mortality based on variables related to survival and worse outcomes.

MATERIALS AND METHODS

We conducted a retrospective study of patients with stage IV who underwent jejunostomy for obstructive GC at the Cancer Institute between 2009 and 2020. The indications for jejunostomy and inclusion in the study were: Adenocarcinoma histology, obstructive GC that could not be resected, diffuse or proximal gastric lesion that could not be submitted to gastrojejunostomy.

Abdominal and pelvis computed tomography (CT), endoscopy, and laboratory tests were assessed preoperatively in all patients. The clinical data collected from our prospectively-maintained database included body mass index (BMI), laboratory blood test, the clinical performance by the American Society of Anesthesiologists (ASA) classification[6], the presence of comorbidities using the Charlson Comorbidity index (CCI)[7] (without the inclusion of age and GC as comorbidity), and *Lauren* histological type. GCs were staged according to the TNM 8th edition.

All patients were operated in a high-volume center by specializes surgeons. Postoperative complications (POC) were graded according to Clavien-Dindo's classification[8] and major POC were defined as Clavien III-V.

Palliative chemotherapy (CMT) consisted of a doublet containing fluoropyrimidine (capecitabine or 5-fluorouracil) and a platin (oxaliplatin or cisplatin) as the preferred systemic regimen for the first line. Irinotecan and cisplatin chemotherapy was chosen in some cases to avoid the use of infusion pumps or in those patients with difficulty swallowing capecitabine tablets. Paclitaxel or irinotecan was used in the second line. In our center monoclonal antibodies (trastuzumab or ramucirumab), as well as immunotherapy, are not usually available for GC treatment[9-11].

Postoperative follow-up was performed every month or in shorter period if necessary. Absence in appointments for more than 12 mo was considered as a loss of follow-up. The study was approved by the hospital ethics committee (NP1681/20) and registered online (https://plataformabrasil.saude.gov.br/; CAAE: 31626220.8.0000.0068).

Prognostic scoring system-predicted mortality

To create a scoring system, patients were randomized into two groups (1:1) by computer using the statistical software (SPSS). The score was developed with half of the patients and further evaluated in a validation cohort with the remaining patients. The score was built with 90-d mortality as the main outcome.

As predictors, 11 preoperative clinical variables were selected and classified in a dichotomous way to define the scoring system categories. Clinical and baseline variables—generally used in clinical practice that reflect the general patient´s status—and oncological variables related to the GC, that would have an impact on the prognosis and survival of patients undergoing palliative care, were selected to compose the score[12-15]. The following variables were used: Age, sex, BMI, ASA, CCI, hemoglobin (Hb) levels, albumin levels, neutrophil-lymphocyte ratio (NLR), tumor size, presence of ascites by tomography, and number of disease sites.

The points assigned to each category were determined by binary logistic regression analysis, and the score was calculated for each patient. The area under the receiver operating characteristic (ROC) curve (AUC) was used to evaluate the performance metric of the score, as explained below.

Statistical analysis

Statistical analyses were carried out using SPSS software, version 20.0 (SPSS Inc, Chicago, IL). Chi-square or Fisher's Exact test, and *t*-test were used to evaluate categorical and continuous variables, respectively. Factors related to 90-d mortality were analyzed by binary logistic regression analysis, and the odds ratios (ORs) with 95% confidence interval (95%CI) were calculated. The score performance metric was determined by the AUC. An AUC > 0.7 was considered to indicate high diagnostic accuracy, given that at least 70% of the patients were classified correctly as the high-risk group for 90-d mortality. The optimal cutoff values were determined by maximizing Youden's index (sensitivity + specificity - 1). Survival was estimated using

the method of Kaplan–Meier, and the log-rank test was employed for comparisons between the curves. Overall survival (OS) was calculated from the date of surgery until the date of death. *P* value less than 0.05 was considered statistically significant.

RESULTS

During the referred period, 363 patients with clinical stage IV GC underwent surgical procedures. Among these, jejunostomy was performed in 80 (22%) patients. The clinical characteristics of all jejunostomy patients are summarized in Supplementary Table 1.

Patients were predominantly male (62.5%) with a mean age of 62.4 (SD ± 12.8, range 24-84.6) years, and a mean BMI of 21.0 (SD ± 3.9). Most tumors affected the middle third of the stomach (67.4%), with a median diameter of 7.2 cm on the longest axis.

The presence of disease in one or more sites, in addition to the primary, was observed in 70% of the cases: 63.7% had peritoneal metastasis and 17.5% had distant metastasis.

After randomization, jejunostomy patients were divided equally into two groups: (J1) score development group; and (J2) score validation group.

Prognostic index and risk groups

Considering the J1 group, the 90-d mortality rate was 52.5%. To develop the predictive score of mortality in 90-d, variables related to the worst outcome were selected for the design of the model. After the binary logistic regression analysis (Supplementary Table 2), 11 variables were included in the model, and points were assigned as shown in Table 1, for a maximum score value of 100. The following variables with the respective defined categories were used: Age (< 65 *vs* ≥ 65 years), sex (female *vs* male), BMI (< 18.5 kg/m^2 *vs* ≥ 18.5 kg/m^2), ASA (I/II *vs* III/IV), CCI (0 *vs* ≥ 1), Hb levels (≤ 11 g/dL *vs* > 11 g/dL, which represents the lower limit between mild and moderate anemia for men and women), albumin levels (< 3.5 g/dL *vs* ≥ 3.5 g/dL), NLR (< 2.5 *vs* ≥ 2.5), tumor size (≤ 7 cm *vs* > 7 cm), number of disease sites (one *vs* two or more, with represents locoregional, distant, or peritoneal) and the presence of ascites by tomography (absent *vs* present).

The highest score value was assigned to the high NLR category and the lowest value to the low levels of Hb and BMI. After, a value was assigned to each of the 40 patients, and the performance metric of the score was assessed through the construction of the ROC curve (Figure 1A). The AUC was 71.2% (AUC 0.712, 95%CI: 0.537–0.887, *P* = 0.022), with an estimated value of 65.5 for optimal cutoff.

Based on the cutoff, the J1 patients were divided in two risk groups for 90-d mortality: Low-risk (score < 65.5), with 14 (35%) patients; and high-risk (score ≥ 65.5) with 26 (65%) patients. The characteristics of the groups are shown in Table 2.

Sex, age, stage, ASA, and the presence of comorbidities showed no statistically significant differences between the groups. High NLR (*P* = 0.001), albumin levels lower than 3.5 g/dL (*P* = 0.026), and tumor size greater than 7 cm (*P* = 0.001) were related to the high-risk group**.** The risk groups showed a significant association with both 30 and 90-d mortality (*P* = 0.013 and *P* < 0.001, respectively).

Internal validation

Using the previously constructed scoring system, the score value was calculated for each patient in the validation cohort (J2 group, *n* = 40). The rate of 90-d mortality in this group was 50%.

As shown in Figure 1B, the risk score had superior diagnostic accuracy for 90-d mortality in the validation cohort (AUC 0.756, 95%CI: 0.598–0.915, *P* = 0.006). Using the previously established cutoff value (65.5 points), 21 (52.5%) and 19 (47.5%) patients were classified as low-risk and high-risk groups, respectively (Table 3).

BMI less than 18.5 kg/m^2 (*P* < 0.001), CCI ≥ 1 (*P* = 0.001), ASA III/IV, (*P* = 0.002), high NLR (*P* = 0.012), and the presence of ascites on CT exam (*P* = 0.004) were significantly associated with the high-risk group.

Considering the postoperative outcomes, there were no differences in POC rate and length of hospital stay between the groups. However, mortality at 30 and 90 d were significantly higher in the high-risk group, demonstrating the performance of the score in predicting mortality based on the adopted cutoff value (*P* = 0.049 and *P* = 0.027, respectively). Besides, the rate of patients who received first and second-line palliative treatment was higher in the low-risk group (*P* = 0.012 and *P* = 0.009, respectively).

Table 1 Variables and points for each category-risk score for 90-d mortality

Variables	Category	Points
Sex	Female	7
Age (years)	≥ 65	4
BMI (kg/m^2)	BMI < 18.5	1
CCI	CCI ≥ 1	7
ASA classification	III/IV	9
Hb (g/dL)	Hb ≤ 11	1
Alb (g/dL)	Alb < 3.5	3
NLR	NLR ≥ 2.5	50
Presence of ascites on CT	Present	5
Tumor size (cm)	> 7 cm	5
Number of disease sites	Two or more	8
Total		**100**

CCI: Charlson's comorbidity index; CT: Computed tomography; NLR: Neutrophil lymphocyte ratio; ASA: American Society of Anesthesiologists; BMI: Body mass index; Hb: Hemoglobin; Alb: Albumin.

Survival outcomes–J1 and J2 groups

The median follow-up time for all patients was 2.1 mo (interquartile range = 0.6-5.9, mean of 4.4 mo). At the time of this study, 8 patients were alive, and 72 patients died (J1 = 36 and J2 = 36). The median OS for the entire jejunostomy cohort was 2.7 mo, compared to a median OS of 8 mo for the other clinical stage IV GC patients undergoing other surgical procedures (resection, bypass, or diagnostic laparoscopy) (P < 0.001) (Supplementary Figure 1).

Regarding the risk groups in J1 patients (development cohort), the median OS for high and low-risk was 1.0 mo and 4.3 mo, respectively (P < 0.001) (Figure 2A). For the J2 patients (validation cohort), the median OS was 1.4 mo for the high-risk compared to 5.7 mo in the low-risk group (P = 0.059) (Figure 2B).

DISCUSSION

In the present study, we performed an analysis of a cohort of stage IV GC, not amenable to surgical resection, who underwent palliative jejunostomy, and we developed a prognostic score for 90-d mortality. Jejunostomy represented the therapeutic choice in 22% of stage IV GC patients. We provided a simple and feasible scoring system with variables easily available in the clinical assessment of patients. The score demonstrated an accuracy of 75.6% in the validation cohort and was associated with mortality rates.

As is widely known, palliative gastric resection is the best option for obstructive lesions, but some patients are unable to undergo this procedure. Proximal and bulky tumors also do not allow gastrojejunostomy to be performed to restore gastrointestinal continuity. Thus, in these cases, jejunostomy or nasoenteric tube are often indicated as a palliative measure to allow maintenance of nutritional enteral support[16]. Once the nutritional emergency is solved, patients may receive palliative CMT and resume oral intake in cases with good response.

The nasoenteric tube causes discomfort in the nasopharynx and oropharynx besides its visual uncomfortable aspect. On the other hand, jejunostomy involves performing an invasive surgical procedure on a patient who is already frail and debilitated[17].

Since these patients have a restricted prognosis, and particular clinical and oncological conditions, the rationale of developing the score was to take into account characteristics that can impact survival to properly identify the patients that are likely to benefit from the procedure.

Building a score involves the appropriate choice of variables since a wide variety of characteristics must be considered in GC cases for the management decision. In our

Table 2 Characteristics of jejunostomy patients according to the risk group-score development cohort (*n* = 40)

Variables	J1-score development cohort		*P* value
	Low-risk	High-risk	
	n = 14 (%)	*n* = 26 (%)	
Sex			0.507
Female	6 (42.9)	14 (53.8)	
Male	8 (57.1)	12 (46.2)	
Age (years)			0.954
mean (SD)	62.0 (15.0)	61.7 (12.9)	
BMI (kg/m^2)			0.101
mean (SD)	20.4 (2.7)	22.4 (3.9)	
CCI			0.885
0	10 (71.4)	18 (69.2)	
≥ 1	4 (286)	8 (30.8)	
ASA class			0.507
I/II	8 (57.1)	12 (46.2)	
III/IV	6 (42.9)	14 (53.8)	
Hemoglobin (g/dL)			0.456
mean (SD)	11.1 (1.9)	10.5 (2.0)	
Albumin (g/dL)			0.123
mean (SD)	3.7 (0.6)	3.3 (0.6)	
NLR			0.001
mean (SD)	3.03 (1.58)	7.46 (5.22)	
Tumor size (cm)			0.18
mean (SD)	6.9 (2.1)	8.3 (3.4)	
Presence of ascites on CT			0.48
Absent	6 (42.9)	7 (26.9)	
Present	8 (57.1)	19 (73.1)	
Lauren type			1
Intestinal	4 (28.6)	6 (23.1)	
Diffuse/mixed	9 (64.3)	17 (65.4)	
Undetermined	1 (7.1)	3 (11.5)	
Number of disease sites			0.408
One (only locoregional)	6 (42.9)	7 (26.9)	
Two or more	8 (57.1)	19 (73.1)	
POC			0.075
No POC/Clavien 1-2	14 (100)	19 (73.1)	
Clavien 3-5	0 (0)	7 (26.9)	
Length of hospital stay (days)			0.452
Median (IQR)	4.5 (1.7-6.3)	5 (2.7-9.5)	
Palliative treatment-1st line			0.257
No	6 (42.9)	16 (61.5)	
Yes	8 (57.1)	10 (38.5)	

30-d mortality			0.013
No	13 (92.9)	13 (50)	
Yes	1 (7.1)	13 (50)	
90-d mortality			< 0.001
No	12 (85.7)	7 (26.9)	
Yes	2 (14.3)	19 (73.1)	

SD: Standard deviation; IQR: Interquartile range; POC: Postoperative complication; CCI: Charlson's comorbidity index; CT: Computed tomography; NLR: Neutrophil lymphocyte ratio; ASA: American Society of Anesthesiologists; BMI: Body mass index.

model, we included 11 common usage variables related to the survival outcomes with broad external validity. The variables were categorized in a dichotomous way, which facilitates their use and reproducibility. Additionally, to provide an internal validation, the cohort was randomized into two samples: One for the construction of the model, and the second for the validation of the model.

Considering the variables that support the score, the impact of the NLR on the outcome was noteworthy, since the highest score was achieved by this parameter. The NLR represents a prognostic marker used in different solid tumors, including GC, and its correlation with survival is widely reported. Furthermore, the incidence of POC and their relation to NLR is also described in GC patients[12,18].

The rationale for this result is likely complex. Neutrophils present a pro-tumor behavior, as they promote angiogenesis, damage DNA, inhibit T-cell activity against tumor cells, and facilitate the metastatic process. Inversely, lymphocytes exert an anti-tumor function when they recognize tumor cell antigens, promoting cytolytic activity against these cells. Thus, the high NLR reflects a proinflammatory systemic status, leading to a protumoral environment that allows rapid tumor progression and development[19]. Therefore, NLR may also reflect a more advanced disease stage.

Regarding the outcome used to assign the score, we chose the 90-d mortality because it is considered a good parameter to reflect the value of a palliative procedure in patients with limited survival. Another outcome option to consider would be the ability to receive palliative CMT in the postoperative setting. However, this variable reflects not only the patient's performance after the procedure, but also their decision to perform palliative CMT. Some patients choose to adopt only best support care. Even so, in the validation court, low-risk patients received more frequently first and second lines of CMT.

Regarding complications related to jejunostomy, most of the reports are related to its performance after major gastrointestinal procedures for postoperative nutritional support[4,5,20,21]. Common surgical complications include loss or obstruction of the tube and local leakage of its content. The findings of some studies have shown that jejunostomy placed during GC resection is associated with increased complications. The analysis of this research included only palliative patients already with limited survival. Therefore, it is difficult to assess the impact of possible complications related to the procedure.

As previously mentioned, it is noteworthy that our score could distinguish low-risk patients, which received significantly more palliative CMT and had better median OS. Wang *et al*[22] analyzed 545 palliative procedures for GC treatment. Of these, 77 were considered as intubation procedures (17 gastrostomies and 60 jejunostomies), with a surgical mortality rate of 23.4%. The median OS in this group was 3.8 mo, and patients who underwent intubation procedures receive less chemotherapy compared to resected patients. In the study performed by Schmidt *et al*[23], the intubation group accounted for 12 out of 110 patients. These patients, combined with 52 other patients who underwent surgical procedures that did not involve the removal of the tumor, had a median OS of 9.2 mo. In our study, the median OS for the entire population was 2.7 mo. These results highlight the limited survival of these patients and the need for a good selection of patients for palliative jejunostomy.

Thus, with the application of the score, we were able to distinguish patients with a more limited survival: A high-risk group, with 1 mo and 1.4 mo in J1 and J2 cohorts, respectively; compared to the low-risk group, with 4.3 and 5.7 mo in J1 and J2 cohorts, respectively. This difference was significant in the development cohort but not in the validation cohort. Perhaps, the presence of one outlier with extremely high survival may have influenced the result in the validation cohort. As the score was developed to

Table 3 Characteristics of jejunostomy patients according to the risk group-score validation cohort (*n* = 40)

Variables	J2-score validation cohort		*P* value
	Low-risk	High-risk	
	n = 21 (%)	*n* = 19 (%)	
Sex			0.721
Female	6 (28.6)	4 (21.1)	
Male	15 (71.4)	15 (78.9)	0.072
Age (years)			
mean (SD)	59.7 (12.4)	66.7 (11.4)	
BMI (kg/m^2)			0.074
mean (SD)	21.4 (3.5)	19.0 (4.7)	
CCI			0.001
0	21 (100)	11 (57.9)	
≥ 1	0 (0)	8 (42.1)	
ASA class			0.002
I/II	16 (76.2)	5 (26.3)	
III/IV	5 (23.8)	14 (73.7)	
Hemoglobin (g/dL)			0.345
mean (SD)	11.4 (2.3)	10.6 (2.4)	
Albumin (g/dL)			0.047
mean (SD)	3.6 (0.6)	3.1 (0.6)	
NLR			0.012
mean (SD)	3.65 (3.45)	8.81 (7.64)	
Tumor size (cm)			0.731
mean (SD)	6.6 (2.6)	6.9 (3.4)	
Presence of ascites on CT			0.004
Absent	14 (66.7)	4 (21.1)	
Present	7 (33.3)	15 (78.9)	
Lauren type			0.753
Intestinal	5 (23.8)	7 (36.8)	
Diffuse/mixed	13 (61.9)	9 (47.4)	
Undetermined	3 (14.3)	3 (15.8)	
Number of disease sites			0.385
One (only locoregional)	7 (33.3)	4 (21.1)	
Two or more	14 (66.7)	15 (78.9)	
POC			1
No POC/Clavien 1-2	19 (90.5)	17 (89.5)	
Clavien 3-5	2 (9.5)	2 (10.5)	
Length of hospital stay (days)			0.455
Median (IQR)	3 (3-7.5)	4 (4-8)	
Palliative treatment-1st Line			0.012
No	5 (23.8)	12 (63.2)	
Yes	16 (76.2)	7 (36.8)	

30-d mortality			0.049
No	18 (85.7)	11 (57.9)	
Yes	3 (14.3)	8 (42.1)	
90-d mortality			0.027
No	14 (66.7)	6 (31.6)	
Yes	7 (33.3)	13 (68.4)	

SD: Standard deviation; IQR: Interquartile range; POC: Postoperative complication; CCI: Charlson's comorbidity index; CT: Computed tomography; NLR: Neutrophil lymphocyte ratio; ASA: American Society of Anesthesiologists; BMI: Body mass index.

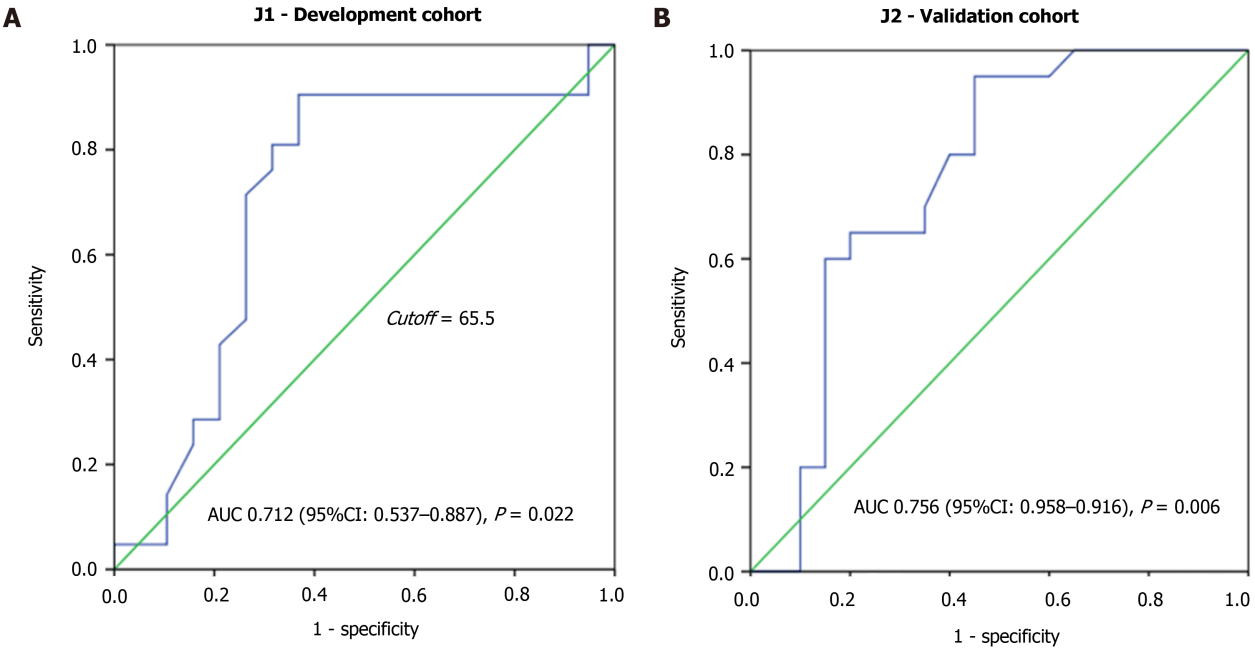


Figure 1 Receiver operating characteristic for the diagnostic accuracy of risk-score. A: Development cohort in predicting 90-d mortality in patients with stage IV gastric cancer (GC) undergoing jejunostomy; B: Validation cohort in predicting 90-d mortality in patients with stage IV GC undergoing jejunostomy. The area under the receiver operating characteristic curve (AUC) was 71.2% in the development group, with an estimated value of 65.5 for optimal cutoff. In the validation cohort the AUC was 75.6%, demonstrating a good diagnostic accuracy for 90-d mortality. AUC: Area under the receiver operating characteristic curve.

predict 90-d mortality, it did not affect the performance of the model. Furthermore, the score performance was higher in the validation cohort compared to the development group (AUC 0.756 *vs* AUC 0.712, respectively).

Some limitations in the present study should be mentioned. Unfortunately, due to its retrospective design, other outcomes related to the effectiveness of nutritional therapy provided by jejunostomy have not been evaluated. Analysis of weight curve, performance status, and quality of life could provide other relevant information. Also, the number of cases is a limitation. Even in a referral center, jejunostomy has a limited role in cancer treatment. This leads to a low volume of reports in the literature concerning its results, and maintains doubts as to its real benefit in cancer care and survival of these patients.

Accordingly, we believe that our series can provide data to assist in the decision of choosing the best way to maintain enteral nutrition. Since our score is easy to perform, it can be applied in other centers—including retrospectively—to evaluate their reproducibility and impact on the oncological outcomes of jejunostomy in these patients.

CONCLUSION

The scoring system developed with 11 variables related to patient's performance status and medical condition was able to distinguish patients submitted to jejunostomy with

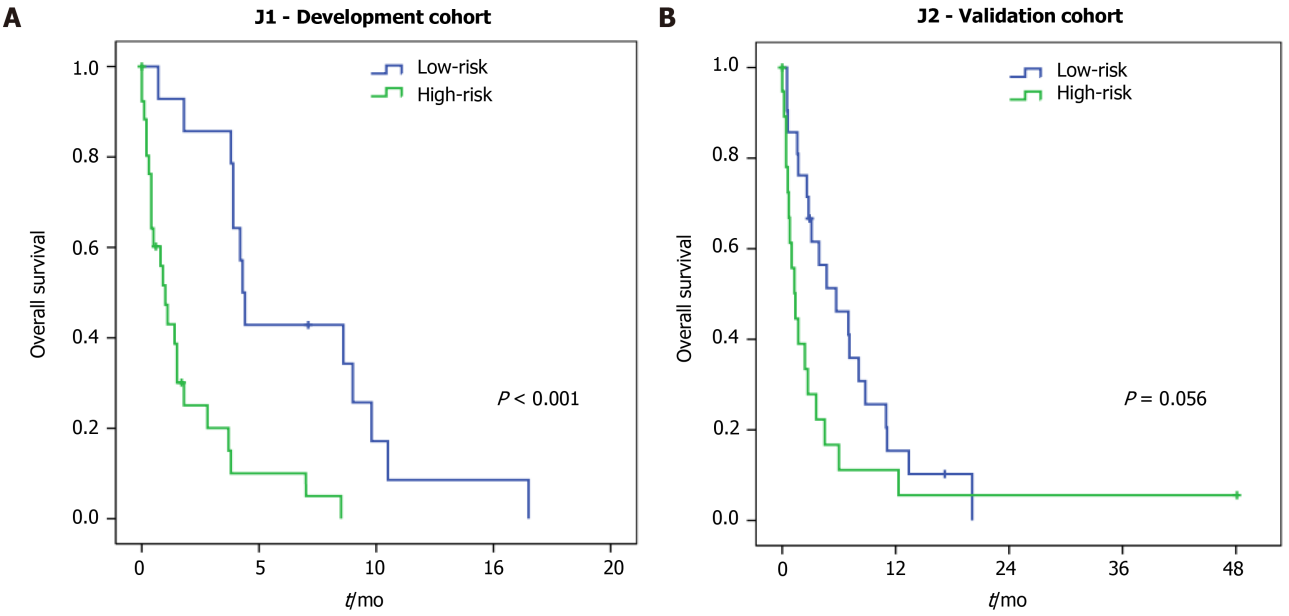


Figure 2 Overall survival of patients with stage IV gastric cancer who underwent jejunostomy according to the risk group. A: Score development cohort; B: Score validation cohort.

a high risk of 90 d mortality. In addition, the score identified patients who were able to receive more CMT in the validation cohort.

ARTICLE HIGHLIGHTS

Research background

Palliative gastrectomy is the initial treatment option for locally advanced gastric cancer (GC) with gastric outlet obstruction whenever it is feasible. Unfortunately, in some cases, nasoenteric tube or jejunostomy becomes the therapeutic alternative to allow maintenance of nutritional enteral support.

Research motivation

The limited survival of these patients raises doubts about who benefits from jejunostomy.

Research objectives

This study aimed to create a prognostic score for 90-d mortality for stage IV GC patients who underwent jejunostomy based on the clinical variables related to survival.

Research methods

We conducted a retrospective analysis of 80 stage IV patients who underwent jejunostomy for obstructive GC. To create a scoring system, patients were randomized into two groups (1:1) by computer using statistical software. The score was developed with half of the patients and further evaluated in a validation cohort with the remaining patients. The score was developed with 90-d mortality as the main outcome.

Research results

We provided a simple and feasible score system with 11 variables easily available in the clinical assessment of patients. The score demonstrated an accuracy of 75.6% in the validation cohort and was associated with the mortality rates in patients who underwent jejunostomy.

Research conclusions

The scoring system developed with variables related to patient's performance status and medical condition was able to distinguish patients submitted to jejunostomy with a high risk of 90 d mortality.

Research perspectives

The results of our series may contribute to identifying stage IV GC with unresectable tumors who can obtain better results with the jejunostomy. In addition, the score may contribute to the selection of patients who were able to receive chemotherapy, and thereby improving their survival.

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ORIGINAL ARTICLE

Observational Study

Novel molecular panel for evaluating systemic inflammation and survival in therapy naïve glioma patients

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Abstract

BACKGROUND

Inflammation is crucial to tumor progression. A traumatic event at a specific site in the brain activates the signaling molecules, which triggers inflammation as the initial response within the tumor and its surroundings. The educated immune cells and secreted proteins then initiate the inflammatory cascade leading to persistent chronic inflammation. Therefore, estimation of the circulating inflammatory indicators kynurenine (KYN), interleukin-6 (IL-6), tissue-inhibitor of matrix-metalloproteinase-1 and human telomerase reverse transcriptase (hTERT) along with neutrophil-lymphocyte ratio (NLR) has prognostic value.

AIM

To assess the utility of chosen inflammatory marker panel in estimating systemic inflammation.

METHODS

The chosen markers were quantitatively evaluated in 90 naive, molecularly subtyped plasma samples of glioma. A correlation between the markers and confounders was assessed to establish their prognostication power. Follow-up on the levels of the indicators was done 3-mo post-surgery. To establish the validity of circulating KYN, it was also screened qualitatively by dot-immune-assay and by immunofluorescence-immunohistochemistry in tumor tissues.

RESULTS

Median values of circulating KYN, IL-6, hTERT, tissue-inhibitor of matrix-metalloproteinase-1 and NLR in isocitrate-dehydrogenase-mutant/wildtype and within the astrocytic sub-groups were estimated, which differed from controls, reaching statistical significance ($P < 0.0001$). All markers negatively correlated

with mortality ($P < 0.0001$). Applying combination-statistics, the panel of KYN, IL-6, hTERT and NLR achieved higher sensitivity and specificity (> 90%) than stand-alone markers, to define survival. The inflammatory panel could discriminate between WHO grades, and isocitrate-dehydrogenase-mutant/wildtype and define differential survival between astrocytic isocitrate-dehydrogenase-mutant/wildtype. Therefore, its assessment for precise disease prognosis is indicated. Association of KYN with NLR, IL-6 and hTERT was significant. Cox-regression described KYN, IL-6, NLR, and hTERT as good prognostic markers, independent of confounders. Multivariate linear-regression analysis confirmed the association of KYN and hTERT with inflammation marker IL-6.There was a concomitant significant decrease in their levels in a 3-mo follow-up.

CONCLUSION

The first evidence-based study of circulating-KYN in molecularly defined gliomas, wherein the tissue expression was found to be concomitant with plasma levels. A non-invasive model for assessing indicators of chronic systemic inflammation is proposed.

Key Words: Circulating; Glioma; Inflammatory marker; Kynurenine; Non-invasive; Prognostic

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Core Tip: The current study is the first-ever analysis of the circulatory levels of kynurenine, interleukin-6, tissue-inhibitor of matrix-metalloproteinase-1 and human telomerase reverse transcriptase along with neutrophillymphocyteratio in a sizeable cohort of molecularly classified glioma samples. The ability of this panel to differentiate survival in glioma subgroups has been assessed. Evaluation of this inflammatory panel of potential biomarkers using the minimally invasive blood sample, for prognostication and targeted personalized therapy in glioma, is suggested.

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INTRODUCTION

The role of inflammation and its partakers in glioma, especially the molecular markers secreted at the tumor initiation stage, is still not completely worked out. Since the brain is unique in comparison to other organ systems, being confined within the blood-brain barrier, it presents a local innate immune response to an inflammatory stimulus, like tumor antigens[1], injury, or oncogene over-expression, by the production of inflammatory molecules and/or metabolites[2]. Consequently, inflammation can be a cause or consequence of actively proliferating cells, an event that also initiates the recruitment of immune cells at the tumor site[3]. The stimuli, if persistent, can manifest as chronic inflammation, aberrant cell proliferation, and increased angiogenesis leading to the escape of specific molecules and cells into peripheral circulation[4]. Therefore, clinical management of inflammation associated with glioma initiation and progression is clinically recognized.

One of the significant inflammatory metabolites in the brain is kynurenine (KYN) of the KYN pathway; it is produced and secreted by endothelial cells and pericytes of the blood-brain barrier, which have been stimulated by inflammation. In the absence of effective immune regulation, chronic inflammation is generated by aggressively proliferating cells. Excess KYN production is triggered by inflammation in the tumor environment (TE), which leads to local immune tolerance, and the inflammatory signals generated by the tumor results in KYN being transduced across the intact blood-brain barrier to be detected in systemic circulation[5,6]. Over-activation of KYN

any other.

Data sharing statement: No additional data are available.

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was first described in glioblastoma (GB) cell lines and tumor tissues as early as 2012 by Opitz and colleagues[7]. Excessive production of this metabolite was linked to increased tumor immunity and decreased survival as recorded by Adams *et al*[8] in cultured cells and 18 GB patient samples[8].

The tumor-infiltrating immune cells - neutrophils and lymphocytes, are also markers of systemic inflammation[9]. Their involvement as local inflammation indicators in glial tumors has been documented by Zadora *et al*[10]. Neutrophil recruitment initiates cytokine secretion, which strengthens the initial *in situ* neuro-inflammatory response by activating more neutrophils and macrophages. Brain tumors, including glioma, express high levels of different cytokines, specifically interleukin-6 (IL-6), involved in multiple pathways of tumor pathology[11]. During gliomagenesis, IL-6 executes a more critical role than other interleukins since it predominately triggers the pro-inflammatory cascade. In the last few years, a couple of blood-based investigations analyzing the expression of IL-6 in different grades of glioma suggested that IL-6 expression may indicate disease prognosis[12,13].

Involved in this scenario of brain inflammation is another set of proteins, the tissue-inhibitor of matrix-metalloproteinases (TIMPs), known to perform multiple functions, including regulating inflammation in the TE. In this context, there is a lone study by Lin *et al*[14] reporting plasma levels of TIMP-1 to be associated with an inflammatory response in glioma, proposing a role of this molecule in prognosis[14].

In this landscape of inflammation partakers in the TE, the association between increased chronic inflammation and telomerase activity is often overlooked. The human telomerase reverse transcriptase (hTERT) is an enzyme active in the immune system cells and regulates inflammation; however, its activity beyond a critical limit initiates uncontrolled proliferation. Several studies suggest that TERT activates the nuclear factor kappa-B target genes such as IL-6, which is crucial to inflammation and cancer progression[15-17]. Research groups, including ours, have demonstrated that hTERT executes many vital functions independent of its telomere maintenance, namely angiogenesis, inflammation, and stemness in glioma[18,19]. These findings suggested that the feedback loop of the hTERT signaling pathway may reinforce the inflammatory signaling *via* cytokine secretion, leading to the development of chronic inflammation in the TE.

Mechanistically, a traumatic event at a specific site in the brain leads to the secretion of inflammatory molecules such as IL-6 and KYN, which trigger the inflammatory cascade leading to over-activation of telomerase. The abnormal hTERT physiology is responsible for immune system dysfunction, followed by persistent chronic inflammation leading to a malignant phenotype. Further, this unresolved chronic inflammation also begins contributing to disease progression. The current exploratory study is the first ever analysis of the circulatory levels of KYN, IL-6, neutrophil-lymphocyte ratio (NLR), hTERT, and TIMP-1 in a sizeable cohort of glioma samples categorized according to their histological grade and isocitrate-dehydrogenase (IDH) expression. The ability of this panel to differentiate survival in glioma subgroups has been assessed.

MATERIALS AND METHODS

Inclusion criteria

Patients with symptoms of recurrent seizures and headache radiologically diagnosed and histopathologically confirmed as glioma grade II, III, or IV.

Exclusion criteria

Patients less than 18 years of age or patients histopathologically confirmed as glioma grade I.

Subjects enrolled for observational study

Ninety treatment-naive samples, presenting with clinical symptoms like recurrent seizures, headache, increased intracranial pressure, and radiological diagnosis of glial tumor, were collected from the in-patient ward of the Neurosurgery department. Informed consent in writing was taken from all the participants of the study cohort (IRB/21/Res/11). Forty-five healthy subjects without any recent clinical history of inflammation or autoimmune disease, were taken as controls for blood samples. Whole blood was collected just before surgery; plasma and serum were separated and preserved at -80°C until further testing for target proteins was undertaken. A follow-up sample of all patients was done 3 mo post-surgery. According to the established lab

protocol, the systemic levels of the marker KYN were correlated with its *in situ* tumor expression by immunofluorescence-immunohistochemistry for validation (Supplementary File Method A).

Dot immune-binding assay

Qualitative screening of circulating markers was done using 20μL of serum after removing high abundance proteins. Subsequently, the samples were loaded onto nitrocellulose membrane and incubated overnight at 4°C. Then the membrane was probed with KYN, IL-6, TIMP-1, and hTERT antibody (1:2000 dilution, Monoclonal, Santa Cruz Biotechnology, United States) for 2 h followed by host-specific respective alkaline-phosphatase-conjugated antibody tagging (Santa Cruz Biotechnology, United States; 1:2500 dilutions) for 1 h. The label was detected using an alkaline-phosphatase substrate (BCIP, Sigma Aldrich, United States), which produced a visible signal corresponding to the concentration of the target protein.

Quantification of circulatory markers

Quantification of the biomarkers in plasma samples was done by enzyme-linked immunosorbent assay (ELISA) for KYN (Creative Diagnostics, United States), TIMP-1 (R&D systems, United States), and hTERT (Elabscience, United States) according to details provided in the instruction manuals. The plasma concentrations were noted in ng/mL for KYN and TIMP-1, and ng/L for hTERT. IL-6 test results were computed from the patients' clinical reports and compared with the baseline values earlier established for this marker in the lab[13]. The standard reference range of each marker was defined and set as per kit insert. Complete blood counts of all enrolled subjects were recorded from the pre-surgery blood profile; the neutrophil and lymphocyte counts were extrapolated into the mathematical formula and calculated[18] to establish the NLR in patients and controls.

Statistical analysis

Non-parametric Kruskal-Wallis test was used to assess the difference between samples of different histological grades for plasma values of all biomarkers. Mann Whitney test was applied on the groups stratified according to IDH status and for marker panel KYN, TIMP-1, NLR, IL-6, and hTERT, to differentiate their significance. Analysis of area under the curve for receiver operating characteristic was performed to define the cut-off values and sensitivity of the markers between the grades and within subgroups that attained ≥ 80% specificity. To establish the diagnostic accuracy of the markers, CombiROC software (https://combiroc.eu) was used, and the predictive probability of the inflammatory panel for prognostication was subjected to receiver operating characteristic analysis. To discern the influence of covariates on plasma maker levels, univariate analysis was carried out to identify the confounding factors, namely, age, site, extent of resection, therapy, KYN, TIMP-1, NLR, IL-6 and, hTERT. Parameters attaining a level of significance in this analysis were entered into the multivariate functionally to create the final model. Furthermore, the Cox proportional regression model was used to compute hazard ratios with 95% confidence interval (CI) to establish the independent status of prognostic markers.

Overall survival (OS), defined as the time from randomization to death from any cause, was considered as a direct measure of clinical benefit to the patient. Patients alive or lost to follow-up were treated as censored. Curves defining total survival period with reference to the identified biomarkers were drawn on Kaplan-Meier estimates and differences compared between IDH-mutant/wildtype (IDH-*m/w*) subgroups for statistical significance using the log-rank test. Spearman's rho coefficient was applied to calculate the correlation of all five markers with OS within histological grades, between IDH-*m/w*, astrocytic-*m/w*, and in terms of KYN, TIMP-1, NLR, IL-6, and hTERT. Multivariate linear regression was performed to describe the association of the four markers with inflammation. Paired *t*-test was performed for pre-operative and 3-mo follow-up samples for the panel. The *P* values of all statistical tests were two-sided ($P < 0.05$).

RESULTS

Plasma samples from healthy controls ($n = 45$) and glioma patients ($n = 90$, IDH-*m:* $n = 60$ inclusive of astrocytic and oligo-component and IDH-*w*, astrocytic $n = 30$) formed the study cohort. Details of patients, relevant demographics, and clinical data are presented in Table 1.

Table 1 Demographic features and concentrations of molecular markers of recruited subjects

No.	Factors	Controls	IDH-mutant	IDH-wildtype
1	Sample size, (*n*)	(*n* = 45)	60	30
2	Sex, M/F	M = 30; F = 15	M = 51; F = 9	M = 25; F = 5
3	Age (yr)	25 (21-50)	35.0 (13-75)	51.5 (25-76)
4	Site	-	Frontal = 37/Non-frontal = 23	Frontal = 12/Non-frontal = 18
5	EOR	-	STR = 38; GTR = 14	STR = 30; GTR = 0
6	OS	-	28.5 (1-158)	6.5 (1-38)

Values presented as median with range. M: Male; F: Female; EOR: Extent of resection; STR: Sub-total resection; GTR: Gross total resection; OS: Overall survival; IDH: Isocitrate dehydrogenase.

Qualitative screening

The presence/absence of KYN, IL-6, TIMP-1, and hTERT in circulation was screened on nitrocellulose membrane by dot immune-binding assay. A colored signal (blue) indicated immune-complex formation and presence of the inflammatory molecule KYN (Figure 1) .The signal intensity of each marker corresponded to the pathological grading of the tumor.

Quantification of biomarkers by sandwich ELISA

Median values of plasma KYN in grade-II, III, and IV were 69.86ng/mL, 112.15ng/mL, 237.318 ng/mL, respectively. Levels of circulating KYN, IL-6, TIMP-1, and hTERT emerged as substantially higher ($P < 0.0001$) with increasing histological grade when the Kruskal-Wallis test was applied. The statistical difference between IDH-*m/w* groups (Table 2) using Mann Whitney test was highly significant for KYN ($P < 0.0001$), NLR ($P = 0.0002$), TIMP-1 ($P = 0.0405$) and hTERT ($P < 0.0001$).

The association of plasma levels (preoperative) of all markers yielded a positive correlation with the grade; however, the best value was observed for IL-6 ($r = 0.64$, $P < 0.0001$, Table 3), while the most significant inverse correlation of KYN ($r = 0.6154$, $P < 0.0001$) was attained with OS (Table 3). When the samples were molecularly stratified, there was an inverse correlation of all biomarkers with survival outcome, being worse for IDH-*w* ascompared to IDH-*m*. The Spearman coefficient for inflammatory markers KYN, TIMP-1, NLR, IL-6, and hTERT was significant and positive for tumor grade, but there was an inverse association with OS, suggesting a poor prognosis with increasing systemic levels of these markers in therapy naïve glioma patients.

The tissue expression of KYN was concomitant with its plasma levels and increased with increasing histological grade (Supplementary Figure 1).

Determination of independence in prognostication

The relation of OS with KYN, TMIP-1, IL-6, NLR, hTERT, age, site, the extent of resection and therapy was calculated using univariate and multivariate Coxregression models to identify the plausible prognostic factors (Table 4). Univariate analysis delineated shorter patient survival to be associated with KYN, IL-6, NLR, TIMP-1, hTERT ($P = 0.0001$), and age ($P = 0.0004$). When multivariate Cox-regression model was applied, higher levels of KYN ($P = 0.0003$), IL-6 ($P = 0.0004$), NLR ($P = 0.0001$) and hTERT ($P = 0.0026$) were found to independently define prognosis. Based on these results, it was assumed that TIMP-1 could not be considered a sensitive marker for inflammation. So, the final maker panel of four markers, KYN, IL-6, NLR, and hTERT, was taken further for validation.

AUROC

The cut-off thresholds based on area under the curve for the four circulatory biomarkers were KYN: > 22.89, IL-6: > 62.5, NLR: > 2.775, and hTERT: > 1.309, at more than 70% sensitivity and 80% specificity, when comparing controls *vs* glioma patients. Levels of KYN, IL-6, NLR, and hTERT could significantly differentiate between histological grades, low and high grade and IDH-*m/w*, with more than 80% sensitivity (Supplementary Table 1).

Based on the AUROC analysis, optimal cut-off points were determined for the best balance of sensitivity and specificity and the highest value of likelihood ratio to predict survival through log-rank analysis, and thereafter Kaplan Meier curves were con-

Table 2 Concentrations of molecular markers of recruited subjects of glioma patients in terms of isocitrate dehydrogenase mutant and wildtype glioma

No.	Factors	Controls (*n* = 45)	IDH-mutant (*n* = 60)	IDH-wildtype (*n* = 30)	*P* value
1	KYN (ng/mL)	5.009 (0.052-34.43)	77.155 (1.56-387.44)	237.318 (53.43-894.95)	< 0.0001[c]
2	IL-6 (pg/mL)	37.009 (6.715-62.49)	68.62 (21.85-209.73)	197.672 (59.217-803.711)	< 0.0001[c]
3	NLR	1.59 (0.87-2.8)	3.67 (1.23-11.5)	5.585 (3.1-18)	0.0002[c]
4	TIMP-1 (ng/mL)	38.03 (10.26-73.99)	82.54 (25.09-131.30)	107.01 (37.175-185.09)	0.0405[a]
5	hTERT (ng/mL)	1.029 (0.075-1.58)	1.364 (0.1-6.886)	2.185 (0.565-8.84)	< 0.0001[c]

[a]*P* < 0.05.
[c]*P* < 0.001.
Median with ranges of all circulatory markers in controls, isocitrate dehydrogenase (IDH)-*m* and IDH-*w*. KYN: Kynurenine; IL-6: Interleukin-6; NLR: Neutrophil-lymphocytes ratio; TIMP-1: Tissue inhibitor metalloproteases-1; hTERT: Human telomerase reverse transcriptase; IDH: Isocitrate dehydrogenase.

Table 3 Spearman coefficient correlation of circulatory markers with overall survival, histological grade and Kynurenine

No.	Circulatory markers	Spearman r	95%CI	*P* value
	Correlation with OS			
1	KYN (ng/mL)	-0.6154	-0.7324 to -0.463	< 0.0001[c]
2	IL-6 (pg/mL)	-0.5531	-0.6854 to -0.3855	< 0.0001[c]
3	NLR	-0.5696	-0.698 to -0.4058	< 0.0001[c]
4	TIMP-1 (ng/mL)	-0.4831	-0.6312 to -0.3011	< 0.0001[c]
5	hTERT (ng/mL)	-0.4386	-0.5960 to -0.2489	< 0.0001[c]
	Correlation with grade			
6	KYN (ng/mL)	0.5409	0.3705 to 0.6761	< 0.0001[c]
7	IL-6 (pg/mL)	0.6400	0.4944 to 0.7507	< 0.0001[c]
8	NLR	0.4982	0.3190 to 0.6430	< 0.0001[c]
9	TIMP-1 (ng/mL)	0.2614	0.05118 to 0.4495	0.0128[a]
10	hTERT (ng/mL)	0.3000	0.06441 to 0.4834	0.0102[a]
	Correlation with KYN			
11	IL-6 (pg/mL)	0.4919	0.3115 to 0.638	< 0.0001[c]
12	NLR	0.4084	0.214 to 0.5717	< 0.0001[c]
13	TIMP-1 (ng/mL)	0.4718	0.2877 to 0.6223	< 0.0001[c]
14	hTERT (ng/mL)	0.3504	0.1485 to 0.5243	0.0007[c]

[a]*P* < 0.05.
[c]*P* < 0.001.
An extremely significant inverse correlation of kynurenine, interleukin-6, neutrophil-lymphocytes ratio, tissue inhibitor metalloproteases-1 and human telomerase reverse transcriptase was obtained with respect to overall survival. A significant positive correlation of interleukin-6, neutrophil-lymphocytes ratio, tissue inhibitor metalloproteases-1 and human telomerase reverse transcriptase was obtained with respect to grades and kynurenine circulatory levels. OS: Overall survival; KYN: Kynurenine; IL-6: Interleukin-6; NLR:Neutrophil lymphocytes ratio; TIMP-1: Tissue inhibitor metalloproteases-1; hTERT: Human telomerase reverse transcriptase; CI: Confidence interval.

structed for the four biomarkers (Figure 2A-D). OS was even better in patients with IDH-*m* astrocytic tumors than their IDH-*w* counterparts (Figure 2E).

Circulating concentrations of KYN, NLR, IL-6 and hTERT were set at 22.89 ng/mL, 2.775, 62.5 pg/mL and 1.309 ng/L, respectively. Patients with values exceeding these thresholds were observed to have a shorter survival period. The OS was defined for KYN (18 *vs* undefined months, HR 3.176 with 95%CI: 1.626-6.206, *P* = 0.0007), NLR (20 mo *vs* 48 mo, HR 0.4167 with 95%CI: 0.204-0.8512, *P* = 0.0025), IL-6 (20 mo *vs* 80 mo,

Table 4 Univariate and multivariate Cox regression analysis of variables

No.	Variable	Univariate		Multivariate	
		HR (95%CI)	*P* value	HR (95%CI)	*P* value
1	KYN (ng/mL)	1.0037 (1.0026-1.0048)	0.0001[c]	1.0024 (1.0011-1.0037)	0.0003[c]
2	IL-6 (pg/mL)	1.0032 (1.0021-1.0042)	0.0001[c]	1.0022 (1.0010-1.0035)	0.0004[c]
3	NLR	1.2437 (1.1621-1.3312)	0.0001[c]	1.2085 (1.1138-1.3114)	0.0001[c]
4	TIMP-1 (ng/mL)	1.0150 (1.0075-1.0225)	0.0001[c]	1.0046 (0.9975-1.0118)	0.2042
5	hTERT (ng/mL)	1.2681 (1.1498-1.3987)	0.0001[c]	1.2303 (1.0749-1.4080)	0.0026[b]
5	Age(yr)	1.0245 (1.0108-1.0383)	0.0004[c]	1.0090 (0.9938-1.0233)	0.2165
6	EOR	1.3379 (1.8805-3.0328)	0.1726	NA	NA
7	Site	0.9527 (0.6221-1.4589)	0.8236	NA	NA
8	Therapy	0.8507 (0.2201-1.3578)	0.906	NA	NA

[b]$P < 0.01$.

[c]$P < 0.001$; indicated a significant association.

Results are presented as estimated hazards ratio with 95% confidence interval for specific variables. Variables with a significant P value (< 0.05) in univariate Cox-regression model were selected and analyzed using multivariate Cox-regression. CI: Confidence interval; EOR: Extent of resection; HR: Hazard ratio; hTERT: Human telomerase reverse transcriptase; KYN: Kynurenine; IL-6: Interleukin-6; NA: Not assessed; NLR: Neutrophil-lymphocytes ratio; TIMP-1: Tissue inhibitor metalloproteases-1.

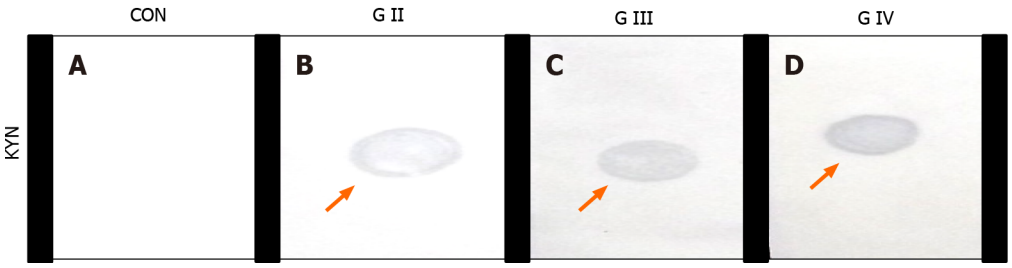


Figure 1 Qualitative screening of kynurenine by dot–immune binding assay in control, grade II, grade III, and grade IV samples; red arrow indicates the presence of immune-complex. A: Control; B: Grade II; C: Grade III; D: Grade IV. KYN: Kynurenine; CON: Control; G: Grade.

HR 0.25 with 95%CI: 0.1318-0.4741, $P = 0.0008$), and hTERT (18 mo *vs* 48 mo, HR 0.21 with 95%CI: 0.1178-0.4741, $P = 0.0006$).

On follow-up, paired t-test between pre-and 3-mo post-surgery levels of KYN ($P = 0.0148$), IL-6 ($P = 0.0107$), NLR ($P = 0.0038$), and hTERT ($P = 0.0016$) showed a significant difference, and lower values post-intervention indicate that inflammation has plausibly reduced on debulking of the tumor (Table 5).

CombiROC

The predicting accuracy of these chosen candidate markers to ascertain systemic inflammation status in glioma patients was increased substantially in combination as a panel rather than standalone markers. Applying CombiROC enhanced the sensitivity of the biomarker panel. The sensitivity of KYN, IL-6, NLR, and hTERT was 86.11%, 72.22%, 77.78%, and 80% individually, which increased to 94.4% in combination with 96.7% specificity and an area under the curve of 0.983 as seen in combination VII (Figure 2F).

Based on the above results and multivariate linear regression, it can be inferred that there exists anassociation between the tumor secreted inflammatory molecules (Figure 3); therefore, these markers can be evaluated to assess systemic inflammation and prognosis.

Table 5 Difference in preoperative and postoperative concentrations of markers in blood samples collected in a 3-mo follow-up

No	Markers	Preoperative concentration	3-mo postoperative concentration	*P* value	rs value
1	KYN (ng/mL)	126.1 (19.410-387.440)	75.405 (16.340-227.400)	0.0148[a]	0.4509
2	IL-6 (pg/mL)	75.65 (23.96-209.73)	60.03 (21.85-140.77)	0.0107[a]	0.1850
3	NLR	4.30 (1.43-15.00)	2.93 (0.80-6.90)	0.0038[b]	0.7260
4	hTERT (ng/L)	2.27 (1.16-6.86)	1.33 (0.78-4.78)	0.0016[b]	0.6574

[a]$P < 0.05$.
[b]$P < 0.01$.
Concentration presented as median with ranges. hTERT: Human telomerase reverse transcriptase; IL-6: Interleukin-6; KYN: Kynurenine; NLR: Neutrophil-lymphocytes ratio.

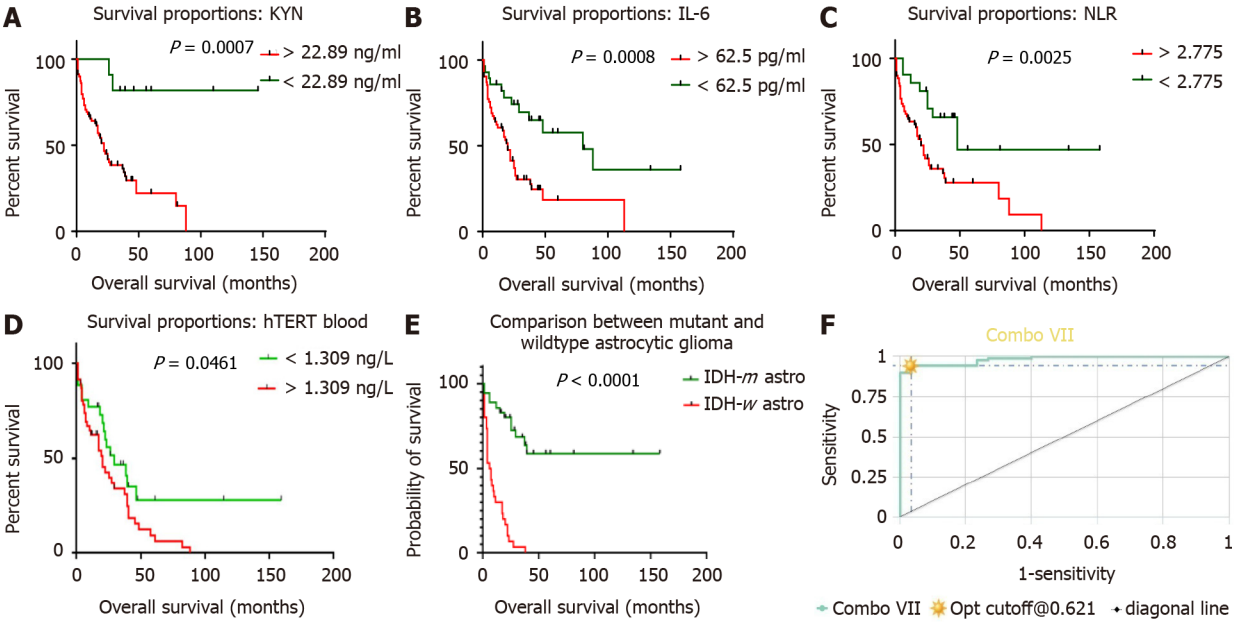


Figure 2 Survival curves of circulatory markers kynurenine, interleukin-6, neutrophil-lymphocyte ratio and human telomerase reverse transcriptase(red indicates worse prognosis while green represents favorable prognosis), differential survival between astrocytic isocitrate dehydrogenase-*mutant* and isocitrate dehydrogenase-*wildtype* gliomas, combination receiver operating characteristic of the panel of kynurenine, interleukin-6, neutrophil-lymphocyte ratio, and human telomerase reverse transcriptase to achieve increased sensitivity and specificity for predicting overall survival. A: Kynurenine; B: Interleukin-6; C: Neutrophil-lymphocyte ratio; D: Human telomerase reverse transcriptase; E: Differential survival between astrocytic isocitrate dehydrogenase-mutant and wildtype gliomas; F: Combination receiver operating characteristic of the panel of kynurenine, interleukin-6, neutrophil-lymphocyte ratio, and human telomerase reverse transcriptase to achieve increased sensitivity and specificity for predicting overall survival. KYN: Kynurenine; IL-6: Interleukin-6; hTERT: Human telomerase reverse transcriptase; NLR: Neutrophil- lymphocyte ratio; IDH-*m* astro: Isocitrate dehydrogenase mutant astrocytic; IDH-*w* astro: Isocitrate dehydrogenase wildtype astrocytic.

DISCUSSION

The link between a tumor and inflammation is a well-established fact and is one of the major attributes of malignancy[19]. Inflammation in the TE mediates all aspects of glial oncogenesis, including *in situ* progressive development of vasculature and tissue remodeling[20]. Thus, by and large, the inflammatory molecules orchestrate the extrinsic and intrinsic stimuli, thereby initiating and contributing to tumor progression [21].

Although KYN is an important inflammatory metabolite in the glial-onco-transformation process, there are limited studies on the role of this molecule in glioma, with three research groups recording the ratio of KYN and tryptophan in a very small number of patients. In reference is the study on the plasma of 18 GB patients by Adams *et al*[8], who presented data to show that because of activation of the KYN pathway, the KYN/TRP-ratio was significantly higher in GB as compared to healthy volunteers. The other group of investigators presented a different opinion on KYN as a marker, stating that there was no significant difference in the levels before and after

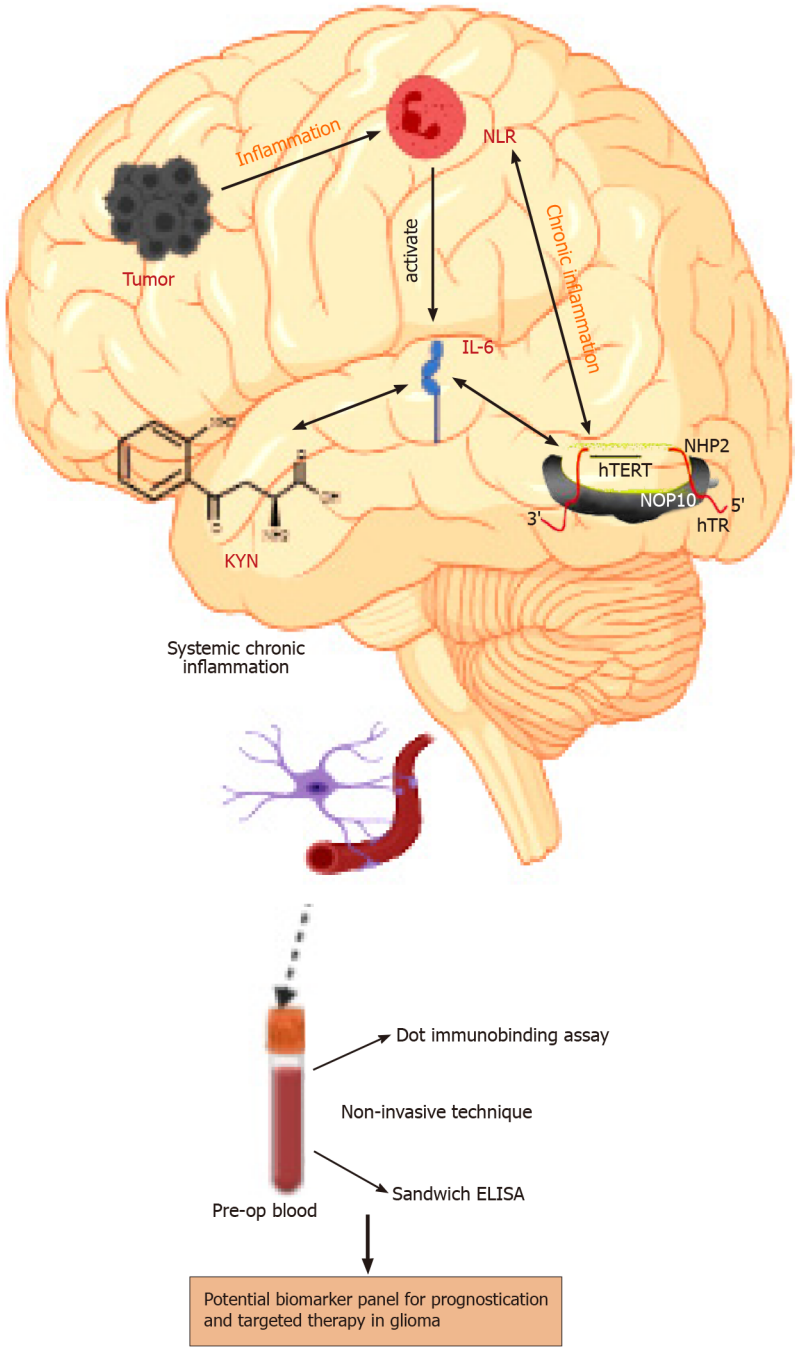


Figure 3 A non-invasive model for evaluating chronic systemic inflammation and overall survival in glioma. KYN: Kynurenine; IL-6: Interleukin-6; NLR: Neutrophil lymphocytes ratio; hTERT: Human telomerase reverse transcriptase; ELISA: Enzyme-linked immunosorbent assay.

surgical intervention, as seen in 10 GB samples[22]. However, the research by Lenzen *et al*[23] documented significantly decreased serological values of KYN after a vaccine treatment with heat shock protein-peptide complex-96, but they did not provide any baseline data of KYN for comparison.

Therefore, the present therapy-naive dataset is unique as it establishes KYN levels in circulation for glioma, with values screened qualitatively at baseline and recorded quantitatively at two time points, pre-and post-surgery. This marker was able to differentiate between IDH-*m/w* groups with high significance (Table 2), along with histological grades and OS (Figures 1 and 2).

When a chronic inflammatory response is generated within the tumor, the KYN pathway proteins trigger the over-expression of pro-inflammatory cytokine IL-6[8]. We noted a raised expression of the inflammatory marker IL-6 in our enrolled glioma patients. Similarly, limited systemic studies available on GB reveal that aberrant production of this circulating cytokine is directly associated with tumor growth and poor survival, demonstrating IL-6 as an independent prognostic factor for survival[13,

24,25]. These studies suggest that the cytokine plays an important role in the *in situ* inflammatory response within the tumor.

Despite the established association between telomerase activity and inflammation, the related molecular pathway in glioma has not been elucidated. Elevated telomerase activity for the sustained proliferation of tumor cells begins a smoldering response of acute followed by chronic inflammation[26,27]. The work of Shervington and Patel[28] in glioma subtypes suggests a noteworthy variation of TERT protein expression levels in GB when compared with control. Two case reports presented from our lab also correlate hTERT expression to proliferation, stemness, and survival in glioma subtypes[13,29]. In a first-of-its-kind investigation, the correlation between tissue and blood concentrations of hTERT marker was also established by us in glioma[30]. Findings of the current analysis are also in line, and there was a significant difference of this marker among grades, IDH-*m*/*w*, and a positive association with KYN and IL-6.

In the present cohort analysis, the NLR score emerged as a good indicator of prognosis and survival in the molecularly typed sub-groups. Concurrence of this result can be found in studies conducted on smaller groups of GB patients[31,32] which show that there is a significant association of NLR with OS[33-36]. Our previous work on diffuse gliomas, along with the current study on IDH characterized gliomas, provides substantial laboratory-based evidence that NLR is a marker of inflammation and can be used as a prognosticator in the clinical setting[18].

There is an increasing acceptance of the proactive role of glial tumor cells in brain inflammation, leading to suppression of innate immune response *via* secretion of matrix metalloproteases[37,38]. In a study conducted earlier in the decade, Sreekantreddy and his co-workers established GBM-specific up-regulation of serum TIMP-1 by ELISA[39]. Similarly, while working with 36 patients, it was suggested by Crocker *et al*[40] that serum TIMP-1 levels may serve as an independent predictor of survival in glioma subtypes[40]. In an extensive study on low-grade glioma patients, Zeng *et al*[41] established that plasma TIMP-1 expression was significantly correlated with OS and relapse-free survival in these patients[41]. However, our results are partially in contention with the above studies. The plasma levels of TIMP-1 were up-regulated in our patients compared to controls, but the marker did not reach significance as an independent prognosticator.

CONCLUSION

The systemic levels of target molecules discussed herein can be considered to represent the cascade of events leading to chronic systemic inflammation in glioma, as depicted in Figure 3. The present work is a one of its kind experiment-based qualitative and quantitative estimation of circulating KYN in a substantial sized cohort of molecularly defined gliomas. KYN is able to differentiate between histological grades, IDH-*m/w*, in terms of patient survival, along with IL-6, hTERT, and NLR, thus advocating assessment of this inflammatory panel of potential biomarkers using the minimally invasive blood sample for targeted therapy and prognostication in glioma. The study needs to be replicated in a bigger cohort and conducted as a multicentric study to establish clinical utility of this panel.

ARTICLE HIGHLIGHTS

Research background

Chronic persistent inflammation is a hallmark of glioma and a major contributor to the disease progression. Currently there are no serological or molecular markers that are routinely evaluated before deciding treatment and in the follow-up period for monitoring survival and therapeutic efficacy.

Research motivation

A non-invasive inflammatory marker panel is essential to define survival and plan for a better clinical outcome.

Research objectives

The objective of this investigation was to assess the utility of the non-invasive biomarker panel to estimate systemic inflammation and whether it can define and differentiate survival in glioma subgroups.

Research methods

Dot-immune assay for screening of the expression of molecular makers followed by estimation of circulatory levels by enzyme-linked immunosorbent assay are easy, cost-effective and sensitive methods even in resource limited settings. The expression of marker kynurenine (KYN) has been validated by immunofluorescence-immunohisto-chemistry *in situ*.

Research results

The molecular marker panel of KYN, interleukin-6, human telomerase reverse transcriptase and neutrophil-lymphocyte ratio were negatively correlated with mortality ($P < 0.0001$) and achieved higher sensitivity and specificity (> 90%) than stand-alone markers, to define survival. It could discriminate between WHO-grades, isocitrate-dehydrogenase-*mutant/wildtype* and define differential survival between astrocytic isocitrate-dehydrogenase-*mutant/wildtype*. Association of KYN with neutrophil-lymphocyte ratio, interleukin-6, and human telomerase reverse transcriptase was significant. Cox-regression described KYN, interleukin-6, neutrophil-lymphocyte ratio, and human telomerase reverse transcriptase, as good prognostic markers, independent of confounders. Multivariate linear-regression analysis confirmed a concomitant significant decrease in the levels of the markers, in a 3-mo follow-up.

Research conclusions

This is a first of its kind evidence-based study of a non-invasive panel that can estimate chronic systemic inflammation, wherein the identified over-expressed molecular markers can be targeted to design a personalized therapy.

Research perspectives

The study model when replicated in a bigger sized multicentric cohort can pave way for the use of the inflammatory molecular screen for routine patient care.

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CASE REPORT

Gastric myeloid sarcoma: A case report

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Abstract

BACKGROUND

Myeloid sarcoma (MS) is a rare hematologic malignancy defined as an extramedullary tumor of immature granulocytic cells. It can occur as primary or *de novo* and be associated with myelodysplasia or myeloproliferative neoplasms. The most frequent locations are the skin, lymph nodes and bones. The case of a patient with a diagnosis of primary granulocytic *de novo* gastric MS is reported.

CASE SUMMARY

A 19-year-old female patient with MS, whose abdominal computed tomography showed a bulky tumor of 16.5 cm in the gastric chamber with infiltration in the retroperitoneal, pancreatic and bile duct region; the histological study showed gastric mucosa diffusely infiltrated by mononucleated cells and the immunohistochemistry expressed myeloperoxidase. After receiving induction chemotherapy based on the 3 + 7 regimen (daunorubicin/cytarabine), the patient developed severe hematological toxicity and neutropenic typhlitis which required a prolonged medical treatment. She presented a rapid disease progression. Although she received supportive treatment, the patient died.

CONCLUSION

Gastric primary *de novo* MS is a rare and aggressive course neoplasm, fostering knowledge is very important to decide its management and to promote more approaches focused on understanding this pathology and its particularities in our population.

Key Words: Myeloid sarcoma; Granulocytic sarcoma; Stomach; Chemotherapy; Peru; Case report

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Core Tip: This case report describes a gastric primary *de novo* myeloid sarcoma (MS) which is a very rare hematological neoplasm with poor prognosis in a young and symptomatic patient. After receiving chemotherapy, she presented severe toxicity (neutropenic typhlitis) and rapid disease progression. This case highlights the importance of detecting gastric primary MS as a rare form of extramedullary myeloid leukemia presentation. Moreover, management of gastric primary MS could lead to interventions to avoid deterioration of gastrointestinal system during treatment. There is limited information of management and outcomes regarding gastric primary MS. Furthermore, there is very limited data about *de novo* MS in Peruvian patients.

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INTRODUCTION

Myeloid sarcoma (MS) is defined as a myeloblast tumor produced in an anatomical place different from the bone marrow that destroys the original architecture of the local tissue. These tumors are also known as granulocytic sarcomas, chloromas or extramedullary myeloid tumors[1,2]. MS occurs more frequently in males and young individuals; its location is variable, and cerebral, mammary, testicular, gastrointestinal involvement has been reported, among other visceral organs, and it appears more frequently in the skin, lymph nodes and bones[3].

MS occurrence is frequently associated with acute myeloid leukemia (AML), affecting between 2.5% and 9% of patients. When the disease is detected without clinical signs of leukemia and in association with a negative bone marrow biopsy, it is classified as *de novo* MS[2], the incidence of which is 2 cases per million adults[2,4]. Gastrointestinal presentation is uncommon and shows nonspecific symptoms related to the effect of tumor mass[3,5].

CASE PRESENTATION

Chief complaints

The patient was a 19-year-old female without a relevant history.

History of present illness

The patient was admitted to the hospital with nausea, vomiting and early satiety of 5 mo of evolution associated with an episode of hematemesis.

Physical examination

The patient showed low body weight, a regular general condition, distended abdomen, ascites, and a poorly defined bulky mass located in the upper hemiabdo-men.

Laboratory examinations

Laboratory tests at admission revealed moderate anemia (Hb: 9 g/dL), hypoalbuminemia (albumin: 2.5 g/dL), grade 4 hyperbilirubinemia (total bilirubin: 2.5 mg/dL, indirect bilirubin: 1.8 g/dL), grade 2 hypertransaminasemia (aspartate aminotransferase: 90 IU/L, alanine aminotransferase: 100 IU/L), and elevated alkaline phosphatase (360 IU/L).

Imaging examinations

Abdominal computed tomography showed a bulky tumor of 16.5 cm in the gastric chamber, which infiltrated the retroperitoneal, pancreatic and bile duct regions, with dilation of the latter. In addition, peritoneal thickening, free fluid, splenomegaly and

bilateral hydronephrosis were observed (Figure 1). Upper digestive endoscopy was reported at the level of the infiltrated gastric mucosal body with decreased contractility, thickened gastric folds, and infiltration of the duodenal bulb throughout its length. The colonoscopy showed no lesions.

Pathology

The histological study showed gastric mucosa diffusely infiltrated by mononucleated cells, which were intermediate in size with eosinophilic cytoplasm and blast-like nuclei (Figure 2). Immunohistochemistry indicated that these cells expressed myeloperoxidase (MPO) (+), CD117 (+) CD34 (+), CD20 (-), CD3 (-), CD68 (-), CD38 (-), CD30 (-), DTT (-), and Ki67: 80% (Figure 3). Bone marrow analysis by cytomorphology and flow cytometry was negative for infiltration by myeloid blasts. The bone marrow karyotype was 46 XX, and it was not possible to demonstrate the presence of the AML1-ETO, CBFB-MYH11, NPM1 mut A, and FLT3-ITD genes or mutations in exons 8 and 17 of the c-kit gene in bone marrow and the primary tumor.

FINAL DIAGNOSIS

This case final diagnosis is primary gastric *de novo* granulocytic MS.

TREATMENT

The patient began induction treatment based on the 3 + 7 regimen (daunorubicin 60 mg/m^2 for 3 d + citarabine 200 mg/m^2 for 7 d). She developed severe hematological toxicity and neutropenic typhlitis requiring antibiotic and antifungal coverage, parenteral nutritional support and a prolonged stay.

OUTCOME AND FOLLOW-UP

In the reassessment of the disease 30 d after treatment, the patient reached a partial response. A second cycle of 3 + 7 regimen treatment was scheduled, and regular tolerance and rapid disease progression were observed, thus she received supportive treatment; however, the patient died.

DISCUSSION

The case of a 19-year-old woman with a final diagnosis of gastric granulocytic type MS is presented. The symptoms were similar to those reported in other cases of gastric MS, which was conditioned by the extensive intra-abdominal involvement of the neoplasm (Figure 1)[2,6,7]. The differential histopathological diagnosis was based on morphological and immunohistochemical characteristics, which included non-Hodgkin lymphoma, Burkitt lymphoma, diffuse large B-cell lymphoma, lymphoblastic lymphoma, and Ewing's sarcoma, among others[3,8]. The present case had a blast type histology. Classically, MS expresses myeloid markers such as CD13, CD33 and MPO, but these markers are not present in all cases. For example, the expression of MPO, one of the most frequent markers, has been reported to be between 83.6% and 64.1% in different series[9]. On the other hand, the expression of CD68, a marker related to lymphocyte lineage and lysosome leakage, was positive in our patient; its presence has been reported in up to 100% of patients with MS[8]. Other markers, such as CD33, CD13 and related to line B, are less consistent in these patients[4,8,9]. It can be distinguished from other round cell neoplasms, such as Ewing sarcoma or neuroendocrine tumors, with specific markers, such as CD99-specific neuronal enolase and CD99, respectively. It has been reported that the anomaly of the nucleus binding factor (translocation between chromosomes 8 and 21) is the most frequent in patients with *de novo* MS; this has been reported in 38% of cases in an North American study[8]. In contrast, translocation 8; 21 was only found in 3% of patients in an Italian series[9]. This translocation was not found in the present case, and there is probably a different frequency of it in our population.

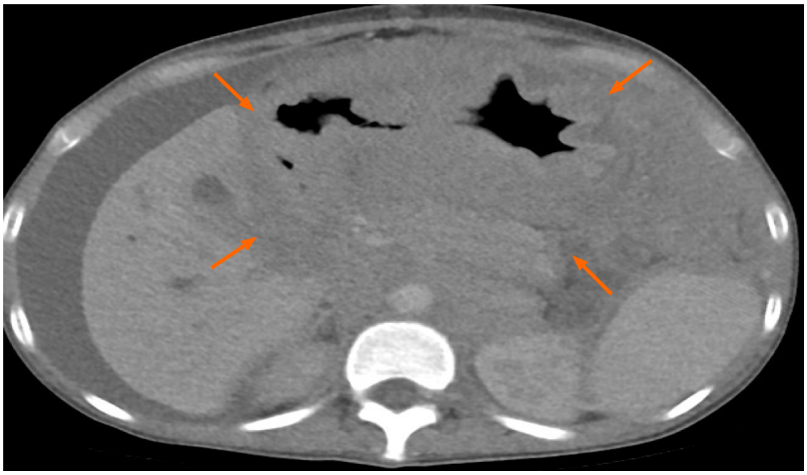


Figure 1 Abdominal computed tomography scan used a part of diagnosis. Computed tomography scan showing extensive intra-abdominal bulky tumor of 16.5 cm at diagnosis with the presence of ascitis.

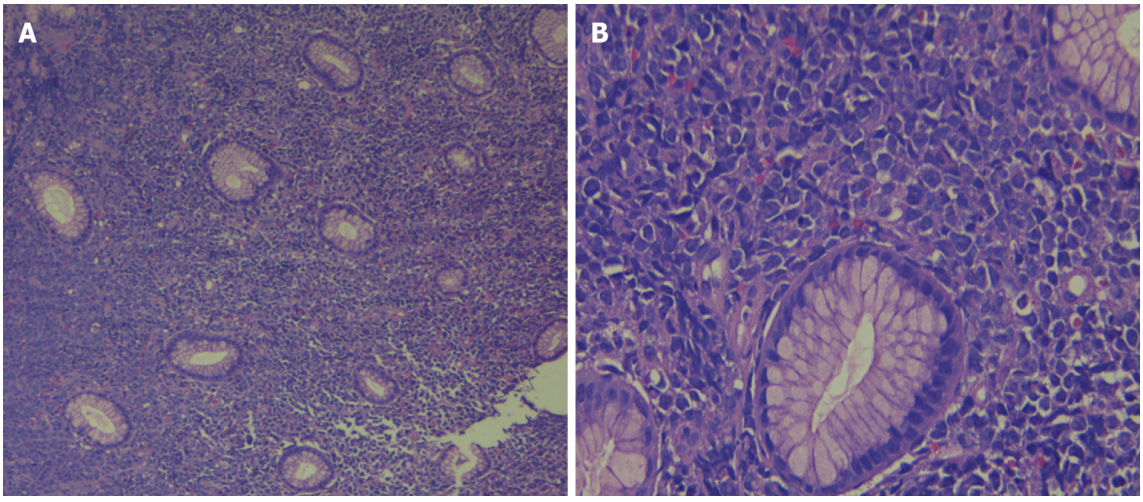


Figure 2 Hematoxylin-eosin staining of gastric myeloid sarcoma. A: There is mucosa with diffuse infiltration of monomorphic medium cells (× 10 magnification); B: At higher magnification (× 40 magnification) the cells show eosinophilic cytoplasm and nucleus with fine chromatin (blast).

Due to the rarity of MS, there are no prospective studies that guide its mana-gement [3,4,8,10], with induction therapy and postadmission therapy for AML being the only current alternatives to treatment. The prognosis of these patients is poor, and a 12-month survival has been reported for patients with MS without treatment and progression within 1 year to leukemia[3]. However, retrospective series such as that of Pileri *et al*[4] and Kawamoto *et al*[9] report that systemic treatment, including daunorubicin and cytarabine, offers longer progression-free survival than local treatments (surgery and radiotherapy). In addition, promising results have been achieved with hematopoietic progenitor (TPH) transplantation; Movassaghian *et al*[10] reported a median OS of 16.7 mo in a retrospective series of 22 patients with allogeneic post-PHT MS. However, despite this, the majority of the patients had disease progression in less than 6 mo[5,7].

CONCLUSION

In conclusion, gastric primary *de novo* MS is a rare neoplasm with an aggressive course. The differential diagnosis depends on the histological and immunohistochemical characteristics. Chemotherapy is the standard treatment, and important results have been reported with bone marrow transplantation. However, further collaborative studies are necessary to understand this pathology and its particularities in our population.

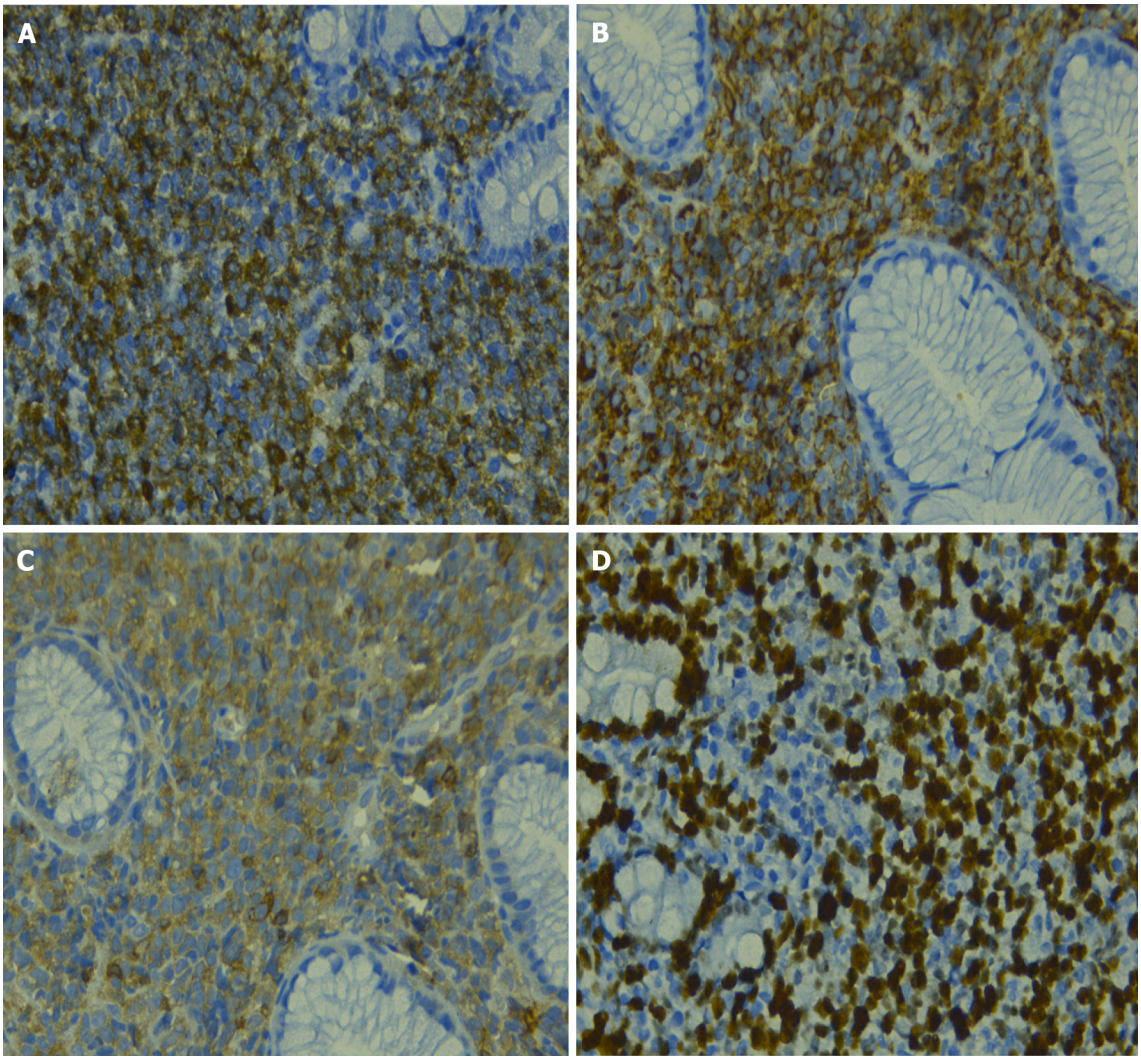


Figure 3 Immunohistochemistry. A: Positive staining for myeloperoxidase (× 40 magnification); B: Positive staining for CD34 (× 40 magnification); C: Positive staining for CD117 (× 40 magnification); D: Strong staining for Ki-67 (proliferation index), around 80% (× 40 magnification).

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REVIEW

Modulation of the immune response by heterogeneous monocytes and dendritic cells in lung cancer

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Abstract

Different subpopulations of monocytes and dendritic cells (DCs) may have a key impact on the modulation of the immune response in malignancy. In this review, we summarize the monocyte and DCs heterogeneity and their function in the context of modulating the immune response in cancer. Subgroups of monocytes may play opposing roles in cancer, depending on the tumour growth and progression as well as the type of cancer. Monocytes can have pro-tumour and anti-tumour functions and can also differentiate into monocyte-derived DCs (moDCs). MoDCs have a similar antigen presentation ability as classical DCs, including cross-priming, a process by which DCs activate CD8 T-cells by cross-presenting exogenous antigens. DCs play a critical role in generating anti-tumour CD8 T-cell immunity. DCs have plastic characteristics and show distinct phenotypes depending on their mature state and depending on the influence of the tumour microenvironment. MoDCs and other DC subsets have been attracting increased interest owing to their possible beneficial effects in cancer immunotherapy. This review also highlights key strategies deploying specific DC subpopulations in combination with other therapies to enhance the anti-tumour response and summarizes the latest ongoing and completed clinical trials using DCs in lung cancer.

Key Words: Lung cancer; Dendritic cell; Monocyte; Tumour microenvironment; Cancer immunotherapy; Dendritic-cell vaccination

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Core Tip: Monocytes and dendritic cells (DCs) as heterogeneous subpopulations may play a key role in the modulation of the immune response in malignant tumours. Monocytes may have a pro- and anti-tumour function and may differentiate into monocyte-derived DC. DCs have the properties of antigen presenting cells. These cells show a different phenotype depending on their maturity and on the influence of the tumour microenvironment. The DCs are of growing interest for their possible beneficial effects in lung cancer immunotherapy. This review highlights specific DC subpopulations in the anti-tumour response and summarizes the latest ongoing DC clinical trials in lung cancer.

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INTRODUCTION

Lung cancer is responsible for approximately 1.8 million deaths annually worldwide and is now one of the most common cancers and the leading cause of cancer mortality [1,2]. The prognosis for lung cancer remains poor despite advances in treatment strategies including immunotherapy with immune check inhibitors (ICIs)[3,4]. Further investigation of tumour immunology and the different cells subpopulations influencing the anti-tumour immune response could enable the development of novel immunomodulatory strategies such as targeted monoclonal antibodies against specific cell receptors.

The results of research on lung cancer show that cells of the same type can have both pro-cancer and anti-cancer properties. Tumour heterogeneity drives a diverse and plastic spectrum of various subpopulations of non-cancer cells. In this review, we focus on assessing different subpopulations of monocytes and dendritic cells (DCs) that may have a key impact on the modulation of immune response in lung cancer.

The role of macrophages, mainly of tumour associated macrophages (TAM) is well recognized. However, the place of monocytes in the anticancer immune response is not fully understoood. We previously presented the results of investigation of macrophages in the direct lung cancer milieu[5] and preliminary studies on monocytes maturation in lung cancer[6]. Monocytes have both pro-inflammatory and anti-inflammatory properties. A phenotype of monocytes can be divided into classical (pro-inflammatory) and non-classical (anti-inflammatory). Both monocyte subpopulations have been detected among the peripheral blood (PB) mononuclear cells and may differentiate into macrophages. Studies demonstrate that monocytes are capable of both inhibition and stimulation of tumour growth[7]. Previous research on monocytes shows that their function in different cancer microenvironments may vary[8,9].

DCs form another heterogeneous population with the most efficient function of antigen presenting cells (APCs)[10]. They take up antigens and pathogens, generate major histocompatibility complex (MHC) peptide complexes, migrate from antigen acquisition sites to secondary lymphoid organs and finally interact with T lymphocytes. DCs infiltrate a tumour, next they process it and then they present tumour-derived antigens to naïve T-cells. DCs play a critical role in priming anti-tumour T-cell immunity and thereby represent a possible therapeutic target for cancer immunotherapy[11].

Moreover, various cell types and factors within the tumour microenvironment (TME) can act on monocytes and DCs, control their differentiation, and affect their biology, function and longevity. The local TME can also influence the activation and the direction of maturation of monocytes and DCs. Specific local microenvironmental factors may influence the formation of monocytes and DCs with tolerogenic or immunosuppressive activity and conversely, specific subpopulations of these cells can stimulate and inhibit the anti-tumour response.

In this review, we summarize the ongoing investigations on monocyte and DCs heterogeneity and function in the context of modulation of the immune response in lung cancer and highlight key strategies using specific monocyte subpopulations and

DCs to improve cancer therapies.

We discuss the heterogeneity of monocytes, their relationship with DCs and the potential of monocyte-derived DCs (moDCs) in the design of vaccines against lung cancer.

HETEROGENEITY OF MONOCYTES

Monocytes are mononuclear immune cells that circulate in PB and direct to tissues at a steady state and at an increased rate during inflammation. Apart from their key role in supporting tissue homeostasis and promoting the immune response to pathogens monocytes take part as regulators of cancer development and progression[12]. As a heterogeneous population, monocytes play opposing roles in inhibiting and stimulating tumour growth and metastases. Monocytes are also precursors of TAM and DCs which are involved in shaping the TME[13]. Monocyte subpopulations perform functions that are involved in both pro- and anti-tumour immunity, including promoting angiogenesis, tumour mediators secretion, phagocytosis, remodelling of the extracellular matrix, influencing lymphocytes, and differentiating into TAM and DCs [14]. Human monocytes express the MHC-II receptor Human Leukocyte Antigen–DR isotype (HLA-DR), integrin αM (CD11b) and CD86. Recent studies demonstrate that monocytes can be divided into three subsets based on the specific surface markers[15, 16]. They develop from the lineage-associated bone marrow (BM) precursor, a common monocyte progenitor (cMoP)[17]. cMoPs are monocyte progenitors that express stem cell marker CD117, C-type lectin CLEC12A, CD64 and CD135, a cytokine receptor and an early hematopoietic marker. cMoP may differentiate into classical monocytes and then convert to non-classical monocytes in the blood, with intermediate monocytes at a transition state[18,19].

These cells perform specific functions: Classical (approximately 85%), intermediate (approximately 5%) and non-classical (approximately 10% of the monocyte population), which are characterized by the degree of CD14 and the expression of CD16[20,21]. There are three types of monocytes in PB: Classical monocytes with high the expression of CD14 cell surface receptor and no CD16 expression (CD14++ CD16-), non-classical monocytes with the low/negative level of CD14 expression and the co-expression of CD16 receptor (CD14- CD16++) and intermediate monocytes with the expression of CD14 and the expression of CD16 (CD14+ CD16+)[22,23]. The majority of non-classical monocytes appears to be derived from classical monocytes. However, the current studies show that there may be a limited progenitor lineage capable of differentiating into non-classical monocytes without classical monocyte origin[19,24, 25]. After differentiation, classical monocytes exit the BM using C-C chemokine receptor type 2 (CCR2) and next migrate into tissues and lymph nodes by l-selectin (CD62L)[26,27]. Monocyte maturation and a scheme of monocyte subpopulations are presented in Figure 1. Classical monocytes and nonclassical monocytes have a different half-life in the circulation: For classical monocytes it is less than 1 d and for nonclassical monocytes it is 7 d[28]. The mechanisms involved in the recruitment of tissue-specific monocytes remain unclear, possibly they depend on the environmental and tissue availability during both homeostasis and inflammation. However, it is known that classical monocytes are more quickly targeted at the site of inflammation and are able to attract other immune cells by secreting cytokines[26,29]. Non-classical monocytes remain in a state of homeostasis mainly in the vascular system and are likely to be able to exit vessels at a slower rate than classical monocytes during inflammation. They are likely to shift into alternative TAMs and exhibit anti-inflammatory properties[28,30].

Different subgroups of monocytes may play various roles in cancer, depending on the tumour growth and progression, differences in the type of cancer, and depending on the influence of TME[14]. Both classical and non-classical monocytes can have pro-tumour and anti-tumour functions. The protumoural phenotype properties consist of: Differentiation into pro-tumoural TAMs, metastatic cell seeding, the suppression of T-cell function, the recruitment of T regulatory cells (Tregs), the promotion of angiogenesis and contribution to extracellular matrix remodelling (ECM)[31,32]. Classical monocytes exit the vasculature to the primary tumour sites using CC-chemokine ligand 2 (CCL2). They produce carcinogenic mediators and are reprogrammed in the TME to limit their cytotoxicity[32]. Then, they differentiate into TAMs or moDCs in the tumour. TAMs are involved in promoting immunosuppression by inhibiting the activity of CD8 T-cells and in stimulating the formation of Tregs[33]. Moreover, they participate in remodelling of ECM and promote angiogenesis[34].

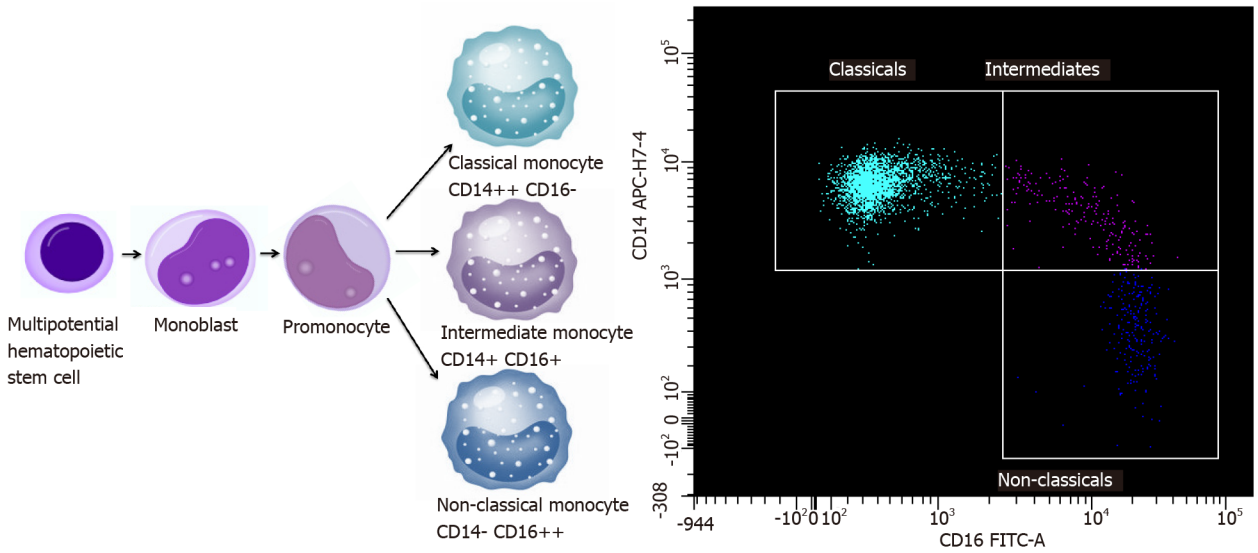


Figure 1 Monocyte maturation and subpopulations scheme. An example of identification of these cells by flow cytometry.

They have a similar protumoural effect at the metastatic sitesand are capable of promoting the spread of metastases. The number of protumoural signals at the tumour site and metastatic sites leads to the predominance of the anti-tumour response from the host's immune system. On the other hand, monocytes have a number of antitumoural functions such as: Antigen presentation, tumour cytotoxicity, the recruitment of natural killer cells, the inhibition of Tregs, the prevention of metastasis[35].

Long-lived non-classical monocytes are well adapted to the removal of cancer cells and debris. Non-classical monocytes migrate towards the sites of cancer spread where they engulf tumour material and produce cytokines that regulate the anti-tumour immunity[36,37]. This population of monocytes could control metastatic process[37]. The third population is a subset of intermediate monocytes, the function of which is under investigation. However, it is known that the relationship between non-classical and intermediate subsets is close[38,39]. The exact maturation and functional relationship between the individual blood monocyte subpopulations and their tissue distribution profiles have yet to be discovered[40,41]. The results of current research confirm that each subpopulation may play a different role depending on the homeostatic and pathological conditions[16]. Infections, inflammation as well as malignant disease can lead to sudden monopoiesis and the formation of a new subset of monocytes with altered functions[35,42].

Monocytes can differentiate into moDCs. MoDCs have a similar antigen presenting ability as classical DCs, including cross-presentation. Blood monocytes can be a reservoir of DC in response to inflammation[43].

HETEROGENEITY OF DCs

Understanding DCs heterogeneity and their role in modulating the immune response in cancer is critical to the better recognition of cancer's ability to bypass the immune system and, consequently, to the ultimate design of novel therapies aiming at boosting anti-cancer immunity.

The studies conducted in order to gain understanding of the biology of DCs have resulted in the identification of a large number of their populations. The main criterion of division is the origin, which distinguishes DCs on plasmacytoid origin cells (pDCs): CD123+ CD11c- and myeloid origin cells: CD123+ CD11c+, also called conventional (cDCs)[44,45]. Identification of antigens called blood DC antigens: BDCA-2, BDCA-3 and BDCA-4 and BDCA-1 (CD1c) allowed further discrimination of human blood DCs into two major subsets: cDC1 and cDC2: cDC1 expresses CD1c, while cDC2 (cCD141+) is characterized by the expression of BDCA-3 (CD141) and Clec9A. BDCA-2 (CD303) and BDCA-4 (CD304), together with CD123, characterize pDC. Additionally, cDCs can be divided into resident and migrating cells[46,47]. DCs derive from the CD34+ hematopoietic stem cell that produces BM myeloid precursors (MPs) and lymphoid precursors (LPs). MPs develop into monocytes, macrophages, and DC precursor

(MDP) from which they differentiate to monocytes and DC precursors (CDP). CDP are precursors of both cDCs and pDCs[48]. Also, cDCs can differentiate directly from monocytes under the influence of various cytokines[49]. Maturation and DC subpopulations scheme is presented in Figure 2.

PDCs

PDCs usually complete their differentiation in BM during the development process and as completely differentiated cells circulate into PB. They are mainly located in the vicinity of the endothelium from where they can easily circulate to the lymph nodes to reside in the T-cell zone[50]. They express the CD123 antigen and the low levels of major MHC-II, the wide range of costimulatory molecules without the expression of CD11c. Additionally, the presence of pattern recognition receptors such as Toll-like receptors (TLRs) allows them to recognize pathogen-associated molecular patterns (PAMP) derived from various microbes and secrete a large amount of type I interferon (INF), tumor necrosis factor (TNF)-α and interleukin (IL)-6[45,51]. What is more, TLR-mediated pDC activation promotes efficient antigen presentation and stimulation of T lymphocytes to the immunological response, but in a less effective manner compared to cDCs[51-53]. Nevertheless, pDCs have also been shown to stimulate Tregs for the production of IL-10, suggesting that this subgroup may also play an immunosuppressive role[54].

CDCs

On the contrary, cDC precursors emerge from the BM transiently transported through the blood and accumulate as cDC pool in tissues[55]. CDCs, also defined as myeloid DCs, expressing CD11c, refer to all DC subsets other than pDC[56]. cCD1c and cCD141 cells belong to the migrating subset of DCs, while epidermal Langerhans cells and interstitial cells are residual cDCs.

CD1c DC subpopulation is the main population of cDCs detected in blood and tissues and lymphatic organs. CD1c cells are identified by major markers CD1c, CD11c, MHC-II (HLA-DR), CD141 and also express other antigens as: CD13, CD33, CD172 and CD45RO. However, they can show a slightly different phenotype, which depends on the place they occur. CD1c+ DCs present in the skin have additional CD1a expression, whereas those present in the gut express CD103[53]. DCs heterogeneity results not only from phenotypic differences but also from the maturity stage. Immature DCs (iDC) are usually found in peripheral tissue, then migrating to the lymph nodes carrying their own antigens, maintaining immune tolerance on their own tissues. They are characterized by increased endocytosis, a decreased expression of MHC and costimulatory molecules and a low ability to produce cytokines[57]. After the antigen is absorbed, the cells begin to mature and change phenotypically and functionally. A peptide complex is formed and an MHC molecule is transported to the cell surface[58]. Maturing DCs migrate to the lymph nodes, increase the expression of MHC-peptide complex, up-regulate the costimulatory molecules and the production of cytokines essential for the T lymphocyte response[55,59]. DCs are considered to be the most important APC, activating T-cells and inducing an immune response. Many factors such as an inflammatory process, an immune response in TME, tissue damage or viruses may promote DCs maturation[60,61].

Many studies show a positive correlation of the number of DCs in the tumour area with a significant extension of patients' survival[62,63]. DCs are able to recognize cancer cells and present neoplastic antigens to effector T lymphocytes. This process depends on the state of maturity and the number of DCs and a local immune status [64].

In fact, amount of data confirm that the accumulation of DCs in the tumour area is influenced by the TME, which modulates their maturation and activation. DCs undergo incomplete differentiation and the number of mature cells decreases with the growth of immature cells[65]. Tumour cells secrete suppressor factors such as transforming growth factor β (TGF-β), IL-10 that reduce the expression of cancer antigens and costimulatory molecules on DCs and that convert them into regulatory DCs (DCreg). DCregs occur among the main cells of the immune system responsible for inhibiting of the immune response, which is conducive to the further development and tumour growth[66,67]. DCs in TME can show an increased expression of programmed death ligand (PD-L1), which interacts with PD-1 molecules on the lymphocytes T surface, inducing their apoptosis, causing the immune response to be muted. DCregs also begin to secrete IL-10, thereby stimulating the proliferation of Tregs and their own polarity to DCregs[68]. Therefore, it seems important to investigate the ways to direct DCs for activating the immune system and inducing an anti-tumour response in an attempt to reverse their suppressive effects. It was

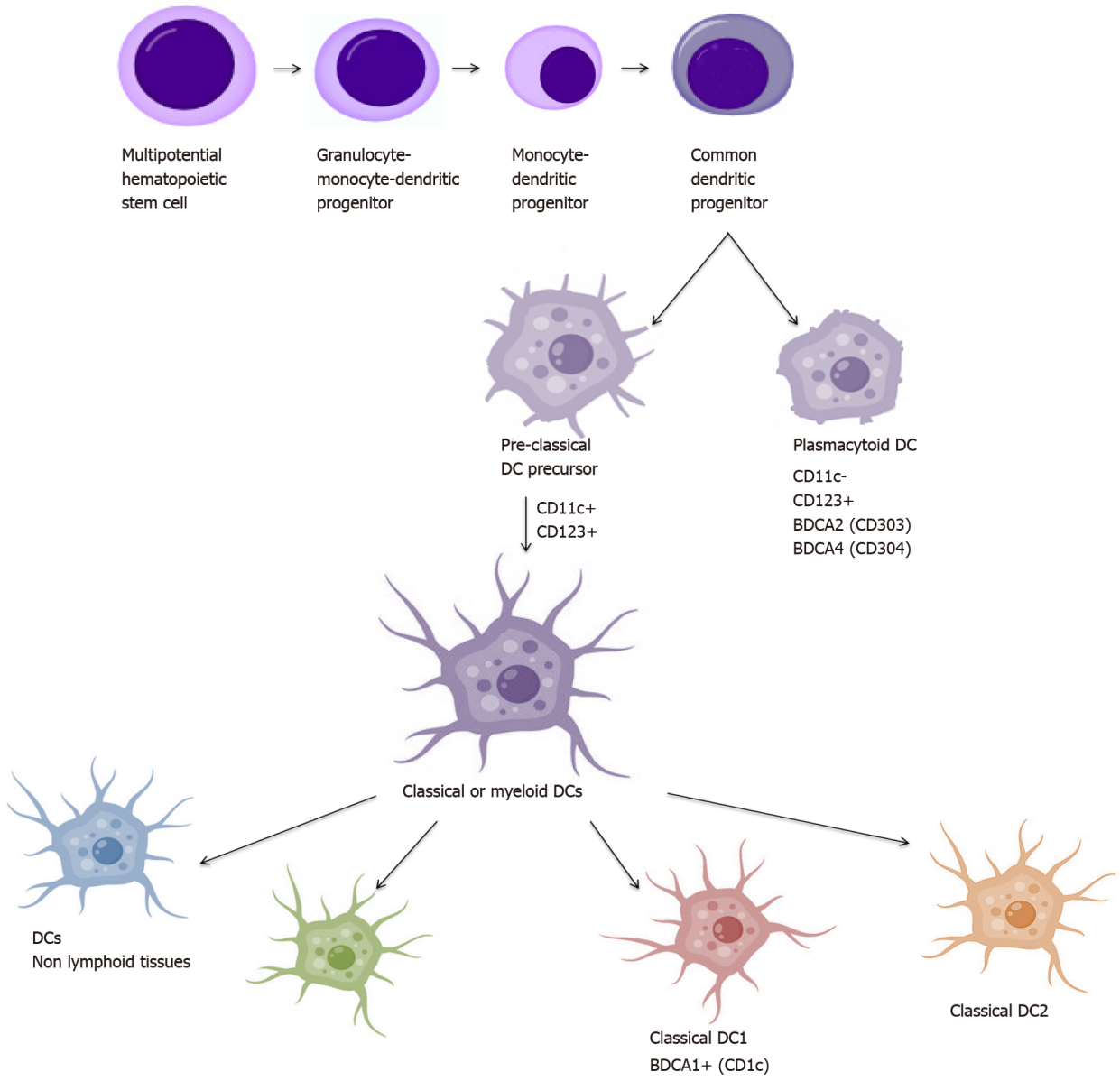


Figure 2 Dendritic cell maturation and subpopulations scheme. DCs: Dendritic cells.

supported by our studies on the identification of DCs in the aspirates from lymph nodes in lung cancer patients. We found an elevated proportion of DCs in metastatic lymph nodes with a high expression of check- point molecules and the phenotype of DCregs.

To sum up, the aforementioned findings confirm the significant participation of DCs in TME. Considering the high heterogeneity of DCs and their plasticity in anti-tumour activity, it seems reasonable to look for a specific subpopulation of these cells.

GENERATION OF moDCs AND APPLICATION IN IMMUNOTHERAPY

Due to the extensive subject matter, we find it valuable to focus on the moDCs population in this review and to discuss DC subpopulation's role in cancer therapy and a possible therapeutic value associated with these populations in lung cancer.

MoDCs arise from monocytes recruited into tissues and become the most abundant DC population during inflammation[69]. In vivo, the maturation of DCs into moDCs is induced by pathogens, tissue damage and cancer antigens. In vitro human moDCs arise from CD14+ monocytes cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. This process is triggered *in vitro* by incubation with pathogen recognition receptor agonists or a pro-inflammatory

cytokines cocktail such as: TNF-α, IL-1β, IL-6, IFN-α and prostaglandin E2 (PGE2) or medium conditioned with monocytes with TNF-α and PGE2[70-72]. TLRs or electroporation with coding proteins have recently been used to induce moDCs maturation [73-75].

Human moDCs always express: HLA-DR, CD11c and frequently express CD16, CD14 due to their monocytic origin upon differentiation[76,77]. Maturation scheme of moDCs is presented in Figure 3.

As an APCs, DCs are crucial in the innate and adaptive response of the immune system and play a crucial role in inducing anti-tumour immunity[78]. Mature DCs present exogenous antigens to naive CD4+ T-cells by MHC-II and endogenous peptides to CD8+ T-cells by MHC-I. What is more, they have the ability to cross-present exogenous MHC-I antigens to CD8+ T-cells, which induces a cytotoxic T-cell response against neoplastic cells[79,80]. MoDC cross-presentation plays a key role in the rapid activation of CD8+ memory T-cells residing in the tissues after infection. This process has been found to be active in immunostimulatory anticancer therapies or chemotherapy[81-84].

In order to stimulate T lymphocytes in lymphoid tissues, it requires three signals between DCs and T lymphocytes. Firstly, the antigen is presented by the MHC peptide complex, secondly stimulation by costimulatory molecules from DC to the T-cell occurs. The third one is the secretion of immunostimulating cytokines in the microenvironment[85-87].

Ex vivo produced moDCs are commonly used in clinical trials. Mature antigen loaded moDCs can be easily obtained from PB derived CD14+ monocytes or a hematopoietic stem and progenitor cell CD34+ by treatment with GM-CSF and IL-4 [88,89]. Multiple clinical trials have demonstrated the safety and immunogenicity of moDC vaccines. However, clinical responses have been largely disappointing. Admittedly, studies show that *ex vivo* produced moDCs were able to cross-prime T-cells and produce anti-cancer cytokines such as IL-12[90-92]. MoDCs seemed to be a promising population in anti-cancer therapy. Although, in clinical practice, only in a few groups of treated patients an active anti-cancer response was achieved. This may be due to functional cell deficiencies in conventional vaccines-such as insufficient antigen presentation, impaired migration, and impaired cytokine release, which is insufficient for gaining a strong immunosuppressive TME[88,93,94]. Some studies show that *ex vivo* stimulation of DCs precursors leads to the production of moDCs that are transcriptionally and phenotypically different from their naturally occurring (primary) cells[92,95]. *Ex vivo* derived moDCs have a reduced ability to stimulate T-cells compared to natural moDCs isolated from PB and may have a limited ability to migrate to lymph nodes, contributing to reduced vaccine efficacy[56,96-98]. All the aforementioned findings explain the lack of efficace vaccine in lung cancer.

APPLICATION DCs IN COMBINATION WITH OTHER THERAPIES

Although, moDCs can be produced in large quantities with minimal side effects from therapy, their effectiveness remains limited in cancer therapy[99,100].

Therefore, other ways of using personalized vaccines with DCs are also being considered. Recent studies show their use in combination with other therapies.

Emerging data suggests that combining DCs vaccination with other cancer treatments could fully unlock the potential of DCs cancer vaccines and improve patient survival. With the advent of combination immunotherapy, personalized DCs vaccination could integrate the current standard of care in the treatment of a wide variety of cancers, among them in lung cancer[88].

The first method is to combine vaccination with chemotherapy to obtain a synergistic effect. In addition to immunosuppressive activity, chemotherapy also strengthens immunity by depleting myeloid derived suppressor cells (MDSCs), Tregs and increases the permeability of cancer cells to cytolytic factors derived from CD8+ T lymphocytes[101,102]. Chemotherapy creates a specific cytokine environment by depleting immune cells, and its combination with DC vaccination and adoptive T-cell transfer has been tested in many trials[103-105]. So far, numerous studies are ongoing in which combination chemotherapy and other methods with DCs vaccines are tested. Chemotherapy with DCs vaccination has been tested with the addition of a Cytochrome c oxidase subunit II inhibitor in patients with melanoma in a phase III trial showing encouraging data. Such a therapy with the addition of autologous T-cells showed longer overall survival compared to chemotherapy alone in two randomized trials in lung cancer[106-108].

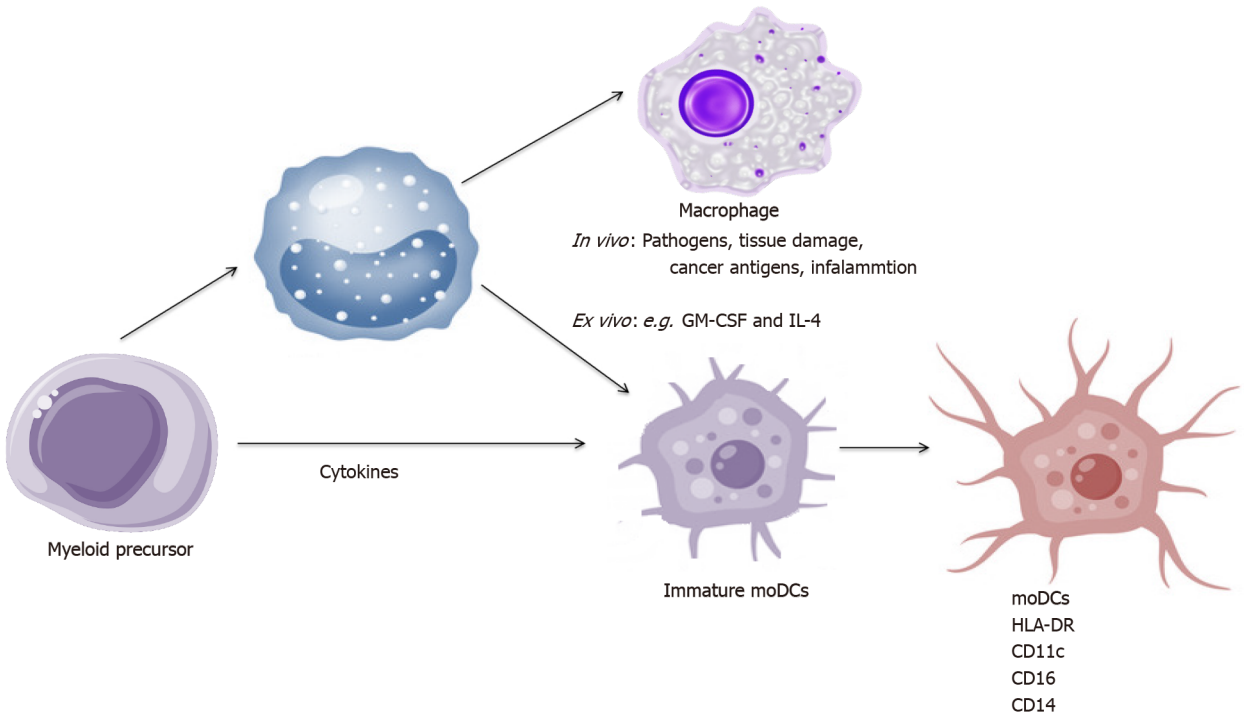


Figure 3 Maturation scheme of monocyte-derived dendritic cells. IL: Interleukin; GM-CSF: Granulocyte-macrophage colony-stimulating factor; DCs: Dendritic cells.

Other treatment method is the combination with immunotherapy. For example, preclinical studies showed that this combination decreased MDSCs in the TME, downregulated the PD-1 expression on DCs, and decreased the secretion of immunosuppressive cytokines[109,110]. One clinical trial in advanced renal cell carcinoma patients showed the expansion of CD8+ T-cells and promising survival data [111].

In general, the combination of DCs vaccines with ICIs seems to be promising. A lot of ICIs are currently being tested in clinical trials; many of them, such as: Anti–PD-L1/PD-1 and anti-*cytotoxic T-cell antigen 4* (CTLA-4) blocking antibodies have been approved by the Food and Drug Administration[112]. The combination of ICIs with the DCs vaccine seems to have the potential to drive T-cell response into a more specific action[113,114]. In addition, DCs unique ability to cross-present antigens helps to elicit the immune response to more cancer antigens when used in conjunction with ICIs[115]. The anti–CTLA-4 treatment after DCs vaccination may indeed enhance DCs vaccine–induced T-cell responses and there is some evidence that anti–CTLA-4 antibodies might be more effective after DCs vaccination[116,117]. Other studies have shown that DC-based immunotherapy in combination with anti–CTLA-4 antibodies seem to be more effective than the use of these agents alone[118,119]. Anti–PD-1 antibodies are being investigated in combination with DC vaccination, which also opens new avenues of anti-tumour therapy design[120]. The aforementioned studies are conducted in various types of cancer, mainly melanoma, pancreatic cancer, prostate cancer, renal cell carcinoma, and acute myeloid leukemia. Immunotherapy with DCs appears to be capable of eliciting strong tumour-specific responses in combination with other therapies, and is workable and safe[121]. In the recent years, the use of naturally circulating DCs (nDCs) instead of cultured moDCs may have represented the next logical step in anti-cancer therapy and had an impact on long-term clinical benefits[83,122,123].

APPLICATION OF DCs IN LUNG CANCER THERAPY

Lung cancer TME is composed of a large number of phenotypically and functionally different types of cells[124]. A major hallmark of immunosuppression in the TME is the inactivation of cytotoxic CD8+ T-cells, which is achieved through diverse pathways[61,125-130]. Immature DCs produce TGF-β, which expands the population of immunosuppressive Tregs, which in turn inhibit CD8+ T-cells. DCs are recruited

Table 1 Clinical trials with dendritic cell vaccine in lung cancer patients on the basis of clinical trials registry

Status	Major trial	Condition	Study intervention	Official trial code
Recruiting	MIDRIXNEO	NSCLC	Dendritic cell immunotherapy	NCT04078269 [132]
Recruiting	MIDRIX4-LUNG	Metastatic NSCLC	Dendritic cell immunotherapy	NCT04082182 [132]
Completed	Vaccine Therapy in Treating Patients With Stage I, Stage II, or Stage III Non-small Cell Lung Cancer	Lung cancer	Autologous dendritic cell cancer vaccine	NCT00103116 [133,134]
Recruiting	Combination Immunotherapy-Ipilimumab-Nivolumab-Dendritic Cell p53 Vac-Patients With SCLC	SCLC; Lung cancer; Relapsed	Combination immunotherapy with Ipilimumab and Nivolumab plus a Dendritic Cell based p53 Vaccine	NCT03406715
Completed	Dendritic Cells in Lung Cancer	NSCLC	Allogeneic Tumour Lysate	NCT00442754
Completed	Chemotherapy Followed By Vaccine Therapy in Treating Patients With Extensive-Stage Small Cell Lung Cancer	Lung cancer	Autologous dendritic cell-adenovirus p53 vaccine combined with Carboplatin and Etoposide	NCT00049218
Completed	Vaccine Therapy in Treating Patients With Stage IIIB, Stage IV, or Recurrent NSCLC	Lung cancer	Autologous dendritic cell-adenovirus CCL21 vaccine	NCT00601094
Completed	CSET 1437	NSCLC	Dendritic cell-derived exosomes	NCT01159288
Completed	Vaccine Therapy in Treating Patients With Stage IIINSCLC	Lung cancer	Mutant p53 peptide pulsed dendritic cell vaccine combined with adjuvant therapy	NCT00019929
Completed	Vaccine Therapy in Treating Patients With NSCLC	Lung cancer	Autologous tumor cell vaccine therapeutic autologous dendritic cells combined with conventional surgery	NCT00023985
Recruiting	AST-VAC2 Vaccine in Patients With NSCLC	NSCLC in the advanced and adjuvant settings	AST-VAC2 Vaccine	NCT03371485
Recruiting	Luscid	NSCLC	Pembrolizumab with or without intratumoral avelumab/ipilimumab plus CD1c (BDCA-1)+/CD141 (BDCA-3) + myeloid dendritic cells	NCT04571632
Completed	To Immunize Patients With Extensive Stage SCLC Combined With Chemo With or Without ATRA	SCLC	Paclitaxel Ad.p53-DC vaccines. ATRA	NCT00617409
Completed	Denileukin Diftitox Followed by Vaccine Therapy in Treating Patients With Metastatic Cancer	Lung cancer; Breast cancer; Colorectal cancer; Pancreatic cancer	Denileukin diftitox recombinant fowlpox-CEA(6D)/TRICOM vaccine therapeutic autologous dendritic cells	NCT00128622
Completed	Biological Therapy in Treating Patients With Metastatic Cancer	Lung cancer; Breast cancer; Colorectal cancer; Extrahepatic Bile Duct cancer; Gallbladder cancer; Gastric cancer; Head and Neck cancer; Liver cancer; Ovarian cancer; Pancreatic cancer	CEA RNA-pulsed DC cancer vaccine	NCT00004604
Completed	Vaccine Therapy and Biological Therapy in Treating Patients With Advanced Cancer	Lung cancer; Breast cancer; Cervical cancer; Colorectal cancer; Ovarian cancer; Pancreatic cancer	Combining DCs vaccine therapy with interleukin-2	NCT00019084

NSCLC: Non small lung cancer; SCLC: Small cell lung cancer; CCL21: Chemokine C-C motif ligand 21; ATRA: All trans retinoic acid.

into the TME and induced to upregulate PD-1 and PD-L1 in order to directly suppress CD8+ T-cells. Interactions of PD-1 with PD-L1 in the TME blocks responsiveness to danger signals and prevents T-cell activation. T-cells are preferentially drawn to tumour induced DCs as they enter the TME. In addition to the lack of appropriate activating signals, T-cell response is blocked by the engagement of PD-1 by PD-L1 on the DC surface[61]. Tregs are also recruited by the tumour induced DCs to establish a tolerogenic environment[124]. TME's effect on DCs infiltrating the lung cancer tissue is presented in Figure 4. A preclinical study conducted by Lee *et al*[131]. showed that the administration of DCs transduced with the chemoattractant CCL21 led to the

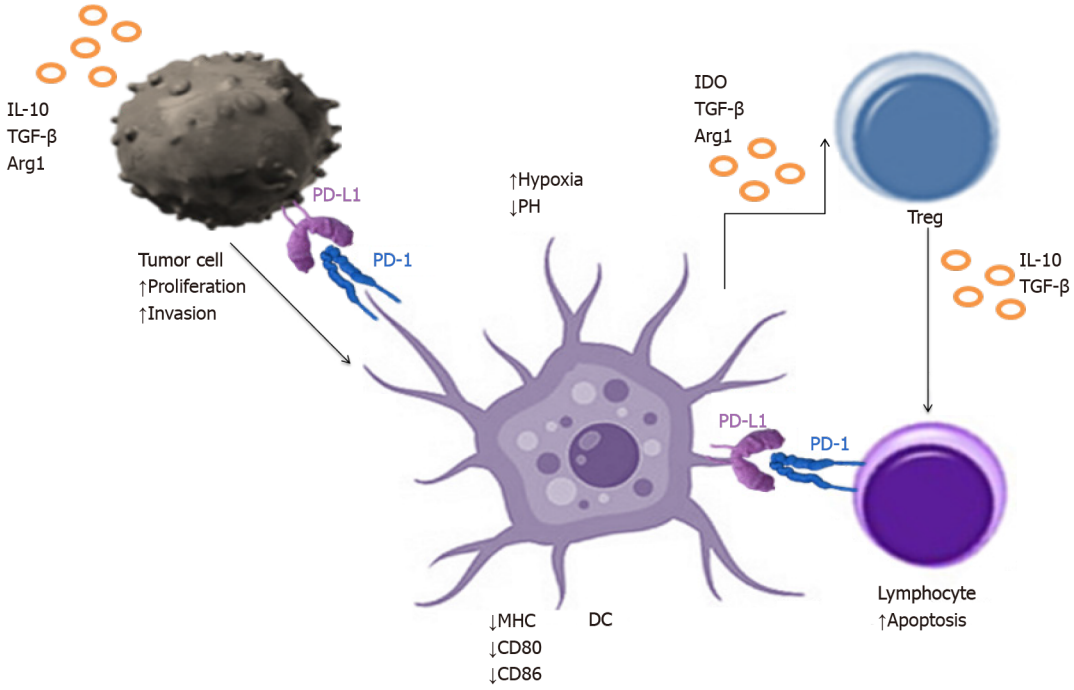


Figure 4 Tumour microenvironment effect on dendritic cells infiltrating the lung cancer tissue. IL: Interleukin; TGF-β: Transforming growth factor β; PD-L1: Programmed death ligand; DCs: Dendritic cells.

increased infiltration of DCs, CD4+ and CD8+ T-cells in the lung TME, resulting in reduced tumour burden[131]. Given that the efficacy of DC vaccines as a monotherapy is limited by immunosuppressive mechanisms in the TME, these results provide a rationale for combining DCs vaccination with immunotherapy. Combinational approach to the lung cancer treatment in order to increase the effectiveness of DCs therapy is an attractive way to promote and stimulate the anti-cancer immunity. As the molecular basis of an effective DCs therapy inducing T-cell response are still incompletely understood, it has been difficult to identify factors associated with therapeutic success. There is also no consensus how DCs vaccination efficacy should be evaluated. However, various clinical trials have been recently conducted to evaluate the immune response and clinical efficacy of DCs in lung cancer and other tumours (Table 1). Unfortunately, it is unknown whether naturally occurring DCs outperform cultured moDCs as a source for DCs therapy in lung cancer patients, because clinical trials comparing different DCs subsets as a source for DCs therapy have not been performed.

CONCLUSION

The heterogeneity of monocytes and DCs has been extensively studied and individual subpopulations of these cells have been well described. As our understanding of monocyte and DCs heterogeneity is growing, their key role as anti-tumour response modulating cells is going to become more useful and targeting the specific subgroups to modulate or stimulate their function is going to become an attractive therapeutic approach. The role of DC in TME is of particular interest in immunological research, but our knowledge is limited, especially in lung cancer. However, the present review emphasizes the role of the DC subpopulation in cancer treatment and a possible therapeutic value associated with these populations in lung cancer. Careful definition of the different subpopulations of DCs and their role in cancer will allow for more accurate targeting of immune cells and a better understanding of their role in modulating the immune response.

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REVIEW

New challenges in the combination of radiotherapy and immunotherapy in non-small cell lung cancer

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Abstract

Immunotherapy has represented one of the main medical revolutions of recent decades, and is currently a consolidated treatment for different types of tumors at different stages and scenarios, and is present in a multitude of clinical trials. One of the diseases in which it is most developed is non-small cell lung cancer. The combination of radiotherapy and immunotherapy in cancer in general and lung cancer in particular currently represents one of the main focuses of basic and clinical research in oncology, due to the synergy of this interaction, which can improve tumor response, resulting in improved survival and disease control. In this review we present the biochemical and molecular basis of the interaction between radiotherapy and immunotherapy. We also present the current clinical status of this interaction in each of the stages and cases of non-small cell lung cancer, with the main results obtained in the different studies both in terms of

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tumor response and survival as well as toxicity. Finally, we mention the main studies underway and the challenges of this interaction in the coming years, including how these treatments should be combined to achieve the greatest efficacy with the fewest possible side effects (dose, type of radiotherapy and drugs, sequence of treatments).

Key Words: Lung cancer; Radiotherapy; Immunotherapy; Main trials

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Core Tip: Immunotherapy has revolutionised cancer treatment. Its association with radiotherapy has synergistic effects studied at a preclinical and clinical level, especially in metastatic patients. Currently, clinical research in this field is very prolific, and no doubt, as with the PACIFIC trial in non-small cell lung cancer (NSCLC), we will see further changes in the standard of care in the coming years. This review highlights the most important published work in NSCLC in the field of radio-immunotherapy, listing the clinical trials currently existing in each stage of NSCLC.

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INTRODUCTION

Immunotherapy, especially in the form of immune checkpoint inhibitors (ICI), is becoming a revolution in the understanding and treatment of cancer. Their interaction with radiotherapy (RT) generates synergistic effects that have been thoroughly described in preclinical studies. In the clinical setting, the benefits of combining RT and ICI have been evidenced mostly in metastatic patients. However, this concept has evolved since the publication of the PACIFIC trial, which has modified clinical practice in unresectable stage III non-small-cell lung cancer (NSCLC) by demonstrating a significant benefit derived from the addition of sequential durvalumab to standard definitive chemoradiotherapy (CRT). Research on the association of RT and ICI in NSCLC is currently an extraordinarily active field with numerous ongoing clinical trials.

IMMUNOTHERAPY IN LUNG CANCER

The development of ICI has become a turning point in the standard of care (SoC) of NSCLC through a significant increase in overall survival (OS) in these patients. ICI were initially approved as second-line therapy for patients who had received combination chemotherapy (CT) including platinum following the results of a number of trials: CheckMate 017 and 057 for nivolumab, Keynote-010 for pembrolizumab and the OAK trial for atezolizumab. These studies showed increased OS in comparison to docetaxel, both in squamous and non-squamous NSCLC[1-4]. In particular, Keynote-10[3] was the first to select patients with a programmed death cell protein ligand 1 (PD-L1) expression in tumor cells ≥ 1%, showing that a higher expression of PD-L1 tends to produce better responses.

In the following years, ICI were tested as first-line treatments. Keynote-024 was the first study to evidence that those patients with advanced NSCLC and PD-L1 > 50% receiving pembrolizumab obtained an OS significantly longer than those treated with a platinum doublet. In the updated analysis, with a median follow-up of 25.2 mo (mo), the median OS was 30.0 mo with pembrolizumab and 14.2 mo with chemotherapy [Hazard ratio (HR), 0.63; 95%CI: 0.47 to 0.86][5,6].

Moreover, the addition of pembrolizumab to platinum plus paclitaxel/nab-paclitaxel *vs* CT alone was assessed in two trials: Keynote-189 (for non-squamous) and Keynote-407 (for squamous NSCLC). A benefit in the pembrolizumab arm was observed in both trials: Progression-free survival (PFS) was 9 mo *vs* 4.9 mo (HR 0.48) and 8 mo *vs* 5.1 mo (HR 0.57), respectively. OS was 22 mo *vs* 10.7 mo (HR 0.56) and 17.1 mo *vs* 11.6 mo (HR 0.71), respectively[7-10]. These benefits were evidenced in all subgroups and were independent of PD-L1 status.

Atezolizumab has also shown good results in the first-line setting. In the IMpower 150 trial, patients with advanced non-squamous NSCLC who received a combination of carboplatin, paclitaxel, bevacizumab and atezolizumab presented higher PFS (8.3 mo *vs* 6.8 mo, HR 0.62) and OS (19.8 mo *vs* 14.9 mo, HR 0.62) when compared to carboplatin plus paclitaxel plus bevacizumab[11]. This benefit was also independent of PD-L1 expression. Interestingly, this has been the only study at this point that included patients with epidermal growth factor receptor and anaplastic lymphoma kinase mutations that had progressed to a tyrosine kinase inhibitor, although OS was not significant in this subgroup. In squamous NSCLC, the IMpower 130 study evidenced that the combination of atezolizumab, carboplatin and nab-paclitaxel improves PFS (7 mo *vs* 5.5 mo, HR 0.64) and OS (13.9 mo *vs* 8.6 mo, HR 0.79) compared to CT alone[12].

In unresectable stage III NSCLC, ICI have also changed the SoC. The results of the PACIFIC trial have led to the approval of one year of consolidative durvalumab in patients who have not progressed after definitive CRT and have a PD-L1 ≥ 1%[13,14].

Finally, in small-cell lung cancer, atezolizumab and durvalumab in combination with a doublet of platinum and etoposide have been recently approved after showing a modest improvement in PFS and OS[15,16].

INTERACTION BETWEEN RADIOTHERAPY AND THE IMMUNE SYSTEM

RT plays a role in all stages of NSCLC, both in the radical and palliative settings[17]. Even though RT has traditionally been considered as an exclusively local treatment, limited to the tissues involved in the radiation field, a number of cases reporting the "abscopal effect" (AE) of RT have been published since its first definition by Mole in 1953, who described it as an antitumor effect taking place outside the radiation field [18]. Recent investigations on this matter have shown that the AE might have an immunological explanation. Several preclinical data have evidenced that RT induces immunogenic cell death through the interaction of tumor-associated antigens (TAAs), damage-associated molecular patterns, high mobility group box 1, heat shock proteins, interferon type I (IFN-I), IFN-γ and other immune mediators[19]. This microenvironment favors the incorporation of TAAs by dendritic cells, which transport these to the lymph nodes in order to present them to naïve T cells through the major histocompatibility complex I. This causes the activation of T cells into cytotoxic T cells, which can then be distributed through the bloodstream and reach distant tumor locations[20,21]. This process is summarized in Figure 1.

For many years, this preclinical data has been difficult to translate into the clinical setting, as reports of the AE have been extremely rare[22]. However, since the introduction of ICI, abscopal responses have become much more frequent, with studies reporting up to 65% rates of AE in patients with metastatic NSCLC and melanoma[23]. This is due to the fact that RT and ICI seem to have a synergistic effect: while ICI "take the brakes off" the immune system by blocking inhibitory signals [such as upregulation of PD-1 and cytotoxic T-lymphocyte antigen 4 (CTLA4)-CD28 inhibition of T cell activation], RT serves as an in-situ vaccination that drives immune cells towards the tumor[24,25].

It must be noted, however, that RT can also unleash immunosupressive effects in certain conditions. A prime example is transforming growth factor beta, which participates in regulatory T cell differentiation. Studies have shown that high levels of this substance are associated with diminished antitumor responses, and its blockade is currently being investigated as a way of improving outcomes[26,27]. Although it is considered that RT generally favors a more immunostimulatory state, more data on various treatment variables that might have an influence on this balance (dose, fractionation, treatment sequence) are still required[28,29].

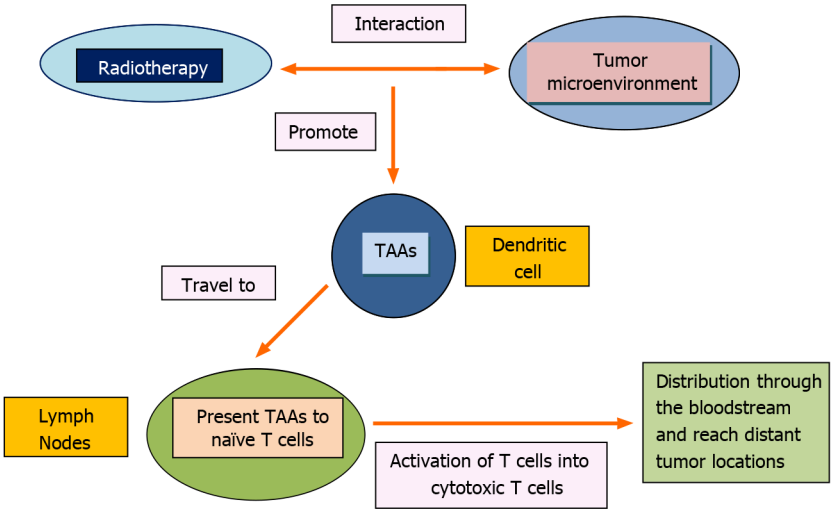


Figure 1 Interaction between radiotherapy and the immune system - Abscopal effect. TAAs: Tumor-associated antigens.

RADIO-IMMUNOTHERAPY IN EARLY-STAGE NSCLC

In the last few years, the results of translational and clinical studies have evidenced the synergistic effects obtainable through the combination of immunotherapy with radiotherapy (iRT) in NSCLC[30,31]. Most of these studies are focused on advanced disease. For instance, this combination has been a paradigm shift in the treatment of unresectable stage III NSCLC following the results of the PACIFIC trial, where an improvement in OS and PFS was observed[13,30].

The management of early-stage NSCLC is generating considerable interest in this setting of combined therapies. Although surgery and stereotactic ablative radiotherapy (SABR) have reported good local control rates (70%-92%) in stage I tumors[32], distant failures can represent up to 30%-60% of cases[33,34]. Moreover, many patients at this stage are inoperable due to comorbidities or refuse surgery. In this scenario, ICI may be an alternative to CT given their comparatively better toxicity profile[31]. The main problem, however, is the poor overall response rate (ORR) of ICI in monotherapy in NSCLC (19% for anti-PD-1 and 4.8% for anti-CTLA-4). For this reason, ICI are being combined with other treatments to achieve better results[35]. Forde *et al*[36] reported a pathological down-staging rate of 40% in patients receiving nivolumab prior to surgical resection (stages I-III). Furthermore, pathological response rates were approximately 10% and no grade ≥ 4 toxicities were observed.

In stage IV NSCLC, iRT in the form of SABR has already achieved an ORR of 36%-41.7% and PFS of 9 mo with a good safety profile[37]. Currently, there are several ongoing clinical trials evaluating iRT in stage I-II NSCLC (Table 1)[38-45]. Although there is still no consensus on the optimal fractionation, ICI agent and treatment sequence, most studies prescribe doses ≥ 6.5 Gy per fraction (fx). The ICI agents include nivolumab, durvalumab, atezolizumab and avelumab. Almost 50% of the registered studies are randomized and evaluate similar primary objectives, such as PFS, local control and toxicity. Among the studies that are already recruiting patients, two of them are randomized phase III studies. The NCT03833159 (PACIFIC-004) trial evaluates sequential durvalumab and SABR in patients with stage I-II NSCLC who are not candidates for surgery. Patients in this study will receive either durvalumab or placebo every four weeks for two years or until treatment discontinuation is necessary [38]. In the NCT04214262, patients will be treated with atezolizumab concurrently with SABR[39]. The exact SABR doses are not specified in either study, but a range of 1-8 fractions will be administered. Great expectations have been placed on these phase III studies, as they could set a new standard in inoperable stage I-II NSCLC.

RADIO-IMMUNOTHERAPY IN STAGE III NSCLC

Stage III NSCLC represents a heterogeneous group of patients with variable prognosis. It includes both resectable tumors in which surgery is the primary curative treatment and CT and RT are administered with neoadjuvant/adjuvant intent, and unresectable tumors in which the SoC is definitive CRT. The suboptimal results of these available

Table 1 Ongoing clinical trials of stereotactic ablative radiotherapy and immune checkpoint inhibitors combination in early-stage non-small cell lung cancer

Ref.	Phase	*n*	Stage	SABR dose	ICI agent	ICI sequence	Status
NCT03833154[38]	III randomized	706	I-II	NM; 3-8 fx	Durvalumab	Sequential	Recruiting
NCT04214262[39]	III randomized	460	I-II	NM; 3-5 fx	Atezolizumab	Concurrent	Recruiting
NCT03110978[40]	II randomized	140	I-IIA	50 Gy/4 fx; 70 Gy/10 fx	Nivolumab	Concurrent	Recruiting
NCT03446547[41]	II randomized	216	I	NM; 3-4 fx	Durvalumab	Sequential	Recruiting
NCT03148327[42]	I-II randomized	105	I-IIA	54 Gy/3 fx; 50 Gy/4 fx; 65 Gy/10 fx	Durvalumab	Concurrent	Recruiting
NCT03050554[43]	I-II	56	I	48 Gy/4 fx; 50 Gy/5 fx	Avelumab	Concurrent	Not recruiting
NCT03383302[44]	I-II	31	I-II	54 Gy/3 fx; 55 Gy/5 fx	Nivolumab	Sequential	Recruiting
NCT02599454[45]	I	33	I	50 Gy/4 fx; 50 Gy/5 fx	Atezolizumab	Induction	Not recruiting

SABR: Stereotactic ablative body radiotherapy; ICI: Immune checkpoint inhibitor; Gy: Gray; NM: Not mentioned.

treatments have led to the investigation of new therapeutic approaches, such as induction/consolidation CT, RT dose escalation, vaccines or targeted therapies. However, none of these have demonstrated significant improvements over standard treatments. For this reason, recent research is evaluating if the incorporation of ICI could improve results, both as consolidation therapy after CRT or surgery, as well as definitive treatment and neoadjuvant therapy.

Neoadjuvant setting

Even though there is currently no evidence on its clinical efficacy, the results of ongoing trials combining ICI with RT prior to surgery in resectable tumors could potentially change clinical practice in the years to come (Table 2). At the moment, there are some data on the combination of ICI with CT. For instance, Forde *et al*[36] reported a 45% of pathological responses in 23 patients with stage I-IIIA NSCLC receiving nivolumab monotherapy, independent of PD-L1 expression. Moreover, the recent NADIM study has observed even higher rates of major pathological response (84.6%) and complete pathological response (71%) with the combination of neoadjuvant nivolumab plus CT[46].

Adjuvant or consolidation setting

The PACIFIC trial was the first randomized double-blinded phase III study to evaluate maintenance durvalumab for 12 mo after definitive CRT in unresectable stage III NSCLC in patients who had not progressed to CRT. A significant increase in PFS (16.8 mo *vs* 5.6 mo) and a manageable toxicity profile (G3-4 30.5% *vs* 26.1%) made this approach the new SoC[13]. Its most recent update in February 2020 reported, with a median follow-up of 33 mo, a 3-year OS of 57% *vs* 43.5% and a 31% reduction in mortality risk[14]. These results were independent of PD-L1 expression, CT regimen and RT dose. In Europe, durvalumab was approved in September 2018, but only in patients with PD-L1 ≥ 1% based on a post-hoc analysis. This debate on PD-L1 status will be addressed in the PACIFIC 5 trial, as the original trial was not designed with this issue in mind[47]. In contrast, the sequence of administration does seem to be relevant, as an improvement in PFS and OS was reported in those patients that started durvalumab within 14 d after CRT.

On the other hand, the LUN 14-179 study, testing consolidative pembrolizumab 4-8 wk after CRT in 93 patients, showed that ICI can also be effective in delivered with a certain delay after concomitant definitive therapy[48]. With a median follow-up of 18.6 mo, the median time to metastatic disease or death was 22.4 mo, while PFS was 17 mo, with a 2-year OS of 61.9%. In regard to toxicity, only 5.4% of patients developed G3-4 pneumonitis.

Consolidative therapy with nivolumab was also being studied in the RTOG 3505 trial (NCT02768558), a randomized phase III study that was prematurely closed after the results of the PACIFIC trial were published[49]. Among the ongoing clinical trials (Table 3), some are investigating dual ICI and the potential side effects of this combination. An interim analysis of the first 20 patients of the NCT03285321 study has reported higher G ≥ 3 toxicity rates in the nivolumab plus ipilimumab arm, but still

Table 2 Ongoing clinical trials of radiotherapy and immune checkpoint inhibitors combination in locally advanced stage non-small-cell lung cancer in the neoadjuvant setting

Ref.	Phase	Design	No.ofpatients	Tumor stage	RT	ICI agent	Sequence	Status
CASE4516, NCT02987998[72]	1	Neoadjuvant CRT (CDDP-etoposide) + ICI followed by surgery and consolidative ICI	20	Resectable IIIA	45 Gy/25 fx (1.8 Gy/fx)	Pembroli zumab	Concomitant (neoadjuvant) + adjuvant ICI	Active, not recruiting
NCT03237377[73]	2	Neoadjuvant RT-ICI followed by surgery +/-adjuvant CT	32	Resectable IIIA	45 Gy/25 fx (1.8-2 Gy/fx)	Durvalumab ± tremelimumab	Concomitant(neoadjuvant)	Recruiting
NCT04245514, SAKK 16/18[74]	2 3 RT arms	Neoadjuvant RT-ICI followed by surgery	90	Resectable IIIA	Randomized 1:1:1; A: 20 × 2 Gy; B: 5 × 5 Gy; C: 3 × 8 Gy (non - consecutive days)	Durvalumab	Concomitant (neoadjuvant)	Recruiting
INCREASE, NL8435[75]	2 single arm	Neoadjuvant CRT (platinum doublet) + ICI followed by surgery	29	Resectable IIB-III (T3-4 N0-1)	50 Gy/25 fx	Ipilimumab + Nivolumab	Concomitant (neoadjuvant)	Recruiting
NCT02904954[76]	2 randomized	Neoadjuvant ICI +/- SBRT followed by surgery and adjuvant maintenance ICI	60	Resectable I-IIIA	SBRT 24 Gy/3 fx	Durvalumab	Concomitantneoadjuvant + adjuvant ICI	Active, not recruiting
NCT03871153[77]	2 single arm	Neoadjuvant CRT (Carbo-taxol) + ICI followed by surgery and adjuvant ICI	25	Resectable IIIA N2	45-61.2 Gy (25-34 fx a 1.8-2 Gy/fx)	Durvalumab	Concomitant (neoadjuvant) + adjuvant ICI	Recruiting
CHIO3, NCT04062708[78]	2single arm	Concomitant neoadjuvant CT (platinum doublet + ICI followed by surgery + adjuvant RT followed by ICI	55	Resectable IIIA-IIIB	54 Gy	Durvalumab	Concomitant CT-ICI (neoadjuvant) + adjuvant ICI (after adjuvant RT)	Not yet recruiting
NCT03102242[79]	2singlearm	Induction ICI followed by definitive CRT (Carbo-Taxol followed by consolidation CT-ICI	63	Unresectable IIIA-IIIB	60 Gy/30 fx	Atezolizumab	Neoadjuvant + consolidative ICI	Active, notrecruiting
NCT02572843[80]	2	Neoadjuvant CT (platinum + docetaxel) + ICI followed by surgery +/-RT + ICI	68	Resectable IIIA N2	Convenional RT if R1-R2 and before adjuvant ICI	Durvalumab	Neoadjuvant + adjuvant	Active, not recruiting

RT: Radiotherapy; CRT: Chemoradiotherapy; CT: Chemotherapy; ICI: Immune checkpoint inhibitor; Gy: Gray.

manageable according to the authors[50].

Definitive setting

Given the good results as consolidation therapy, concomitant ICI with definitive CRT is being investigated in order to further improve clinical outcomes while maintaining an adequate toxicity profile. This has been the case for nivolumab and atezolizumab in the ETOP NICOLAS and DETERRED trials, respectively.

The PACIFIC2 phase III study with durvalumab and the KEYNOTE-799 study with pembrolizumab (Table 4) put a focus on toxicity after the previous studies with nivolumab[51] and pembrolizumab[52], which offered promising outcomes but with an increased risk of pneumonitis. In the first one, the ETOP NICOLAS phase II trial [51], nivolumab is added to standard CRT both as concomitant and consolidation therapy. An interim analysis of the initial 21 patients showed a 1-year OS of 79% with

Table 3 Ongoing clinical trials of radiotherapy and immune checkpoint inhibitor combination in locally advanced stage non-small cell lung cancer in the adjuvant/consolidation setting

Ref.	Phase	Design	No.of patients	Tumor stage	RT	ICI agent	Sequence	Status
BTCRC-LUN16-081, NCT03285321[50]	2 randomized	Concomitant definitive CRT followed by consolidative ICI (3 CT regimens: CDDP-VP16 *vs* Carbo-Taxol *vs* Cisplatin- Pemetrexed)	108	Unresectable IIIA-IIIB	59.4-66.6 Gy	Nivolumab +/- Ipilimumab	Consolidation afterdefinitive treatment	Recruiting
NCT03589547[81]	2	CRT followed by consolidative ICI and SABR	25	III	60 Gy followed by SBRT 20 Gy/2-3 fx	Durvalumab	Consolidation after definitive treatment (ICI prior to SABR)	Recruiting
PACIFIC 6, NCT03693300[82]	2	ICI after sequential CRT	150	Unresectable III	Conventional RT; 60 Gy/30 fx	Durvalumab	Consolidation after definitive treatment (within 28 d after RT)	Active, not recruiting
MK-3475, NCT03379441[83]	2	Maintenance ICI after definitive CRT	126	Unresectable IIIA-IIIB	Conventional RT	Pembrolizumab	Consolidation afterdefinitive treatment	Not recrutiing
DUART,NCT 04249362[84]	2 single arm	RT followed by ICI	150	Unresectable III	Conventional RT 60 Gy Hypofractionated RT 40-54 Gy	Durvalumab	Consolidation after RT (no CT)	Recruiting
PACIFIC 5, NCT03706690[47]	3 randomized, doube-blinded	Consolidative ICI *vs* placebo after definitive CRT radical (concomitant or sequential)	360	Unresectable III	Conventional RT	Durvalumab	Consolidation after definitive treatment	Recruiting

RT: Radiotherapy; CRT: Chemoradiotherapy; CT: Chemotherapy; ICI: Immune checkpoint inhibitor; Gy: Gray.

no G ≥ 3 pneumonitis, which led to the recruitment of additional patients up to a total number of 80. In this case, the analysis of these 80 patients evidenced 10% G ≥ 3 pneumonitis. 1-year OS in this new cohort has not been published yet.

The phase II trial DETERRED[53] with concomitant atezolizumab has completed recruitment and reported better PFS in the ICI arm (57% *vs* 50%), with no significant increase in toxicity but with no benefit in OS at this point (79% in both arms).

In addition, several ongoing studies are investigating if CT can be excluded from radical treatment by combining RT and ICI. The SPRINT trial[54] is evaluating the efficacy of induction pembrolizumab in monotherapy and RT in patients with PD-L1 ≥ 50%. Moreover, the DART study[55] is testing the safety and efficacy of concomitant RT and durvalumab followed by consolidative durvalumab in patients who are not candidates for CT. Other strategies include combinations of ICI with different mechanisms, such as anti-CTLA-4 in the NCT03663166 study (Table 4).

RADIO-IMMUNOTHERAPY IN STAGE IV NSCLC

As mentioned above, the irruption of ICI has been shifted the treatment paradigm in metastatic NSCLC by increasing survival both as first and second-line therapy[2,3,5,7,

Table 4 Ongoing clinical trials of radiotherapy and immune checkpoint inhibitor combination in locally advanced stage non-small cell lung cancer in the concomitant setting

Ref.	Phase	Design	No.of patients	Tumor stage	RT	ICI agent	Sequence	Status
ARCHON-1, NCT03801902[85]	1; 2 RT arms	ICI + RT	24	Unresectable II-III	Conventional RT (60 Gy/30 fx); Hypofractionated RT (60 Gy/15 fx)	Durvalumab	Concomitant with definitive RT	Recruiting
PARTICLE-D, NCT03818776[86]	1; 2 RT arms (proton beam therapy	ICI + RT	27	Unresectable III	RT 60 Gy/20 fx; RT 63 Gy/23 fx	Durvalumab	Concomitant with definitive RT	Recruiting
NCT04013542[87]	1	ICI + RT	20	II-III	Conventional RT	Ipilimumab + Nivolumab	Concomitant with definitive RT and consolidation (nivolumab)	Recruiting
NCT03663166[88]	1; 2	Concomitant CRT + ICI +/- consolidative ICI	50	Unresectable III	60 Gy/30 fx	Ipilimumab +/- Nivolumab	Concomitant definitive treatment +/- consolidative ICI	Recruiting
SPRINT,NCT03523702 [54]	2	ICI + RT (if PD-L1 ≥ 50%) ; CRT (if PD-L1 < 50%)	63	Unresectable III	Conventional RT	Pembrolizumab	Concomitant with definitive RT	Recruiting
KEYNOTE-799, NCT03631784[89]	2	Concomitant ICI + CRT (platinum doublet) followed by ICI	216	Unresectable III	60 Gy/30 fx	Pembrolizumab	Concomitante and consolidative	Active, not recruiting
NCT04092283[90]	3 randomized	Concomitant CRT + ICI *vs* CRT followed by ICI (CT: Cisplatin + VP16/ Taxol + Carboplatin/ Cisplatin + Pemetrexed)	660	Unresectable III	60 Gy/30 fx	Durvalumab	Concomitant with definitive treatment *vs* adjuvant ICI	Recruiting
PACIFIC 2, NCT03519971[91]	3 randomized, double-blinded	ICI *vs* placebo concomitant to CRT (CDDP-VP16 *vs* Carbo-Taxol *vs* Cisplatin or Carbo + Pemetrexed)	328	Unresectable III	Conventional RT (60 Gy in 30 fx)	Durvalumab	Concomitant +/- consolidative	Active, not recruiting
NCT04026412[92]	3 randomized	ICI (nivolumab) + CRT followed by ICI (nivolumab + ipilimumab) *vs* ICI (nivolumab) + CRT followed by ICI (nivolumab) *vs* CRT followed by durvalumab	1400	Unresectable/inoperable III	Conventional RT	Nivolumab; Ipilimumab; Durvalumab	Concomitant + 2 consolidation regimens *vs* consolidation after CRT	Recruiting

RT: Radiotherapy; CRT: Chemoradiotherapy; CT: Chemotherapy; ICI: Immune checkpoint inhibitor; Gy: Gray.

11,56,57]. Furthermore, RT in stage IV has evolved from a merely palliative intent to having a key role when associated with ICI. Reports of objective responses in distant locations not included in the radiation field (AE) have multiplied in the era of immunotherapy[31,58].

Preclinical and clinical data suggest that the antitumor efficacy of ICI increases when combined with RT, with a possible impact in survival, which could be the base for designing new combinations that can maximize this synergistic effect[58-65]. Even though most published studies on the combination of RT and ICI in stage IV NSCLC

are phase I/II trials with a limited number of patients (Table 5), they have laid the foundation for the possible benefits of this strategy that are being determined in ongoing phase III studies.

Although retrospective data had been the only evidence available for years, phase I studies evaluating the safety of the combination recently started to surface. For instance, Tang *et al*[66] treated 21 patients with pembrolizumab and RT (SABR or hypofractionated RT) and reported an ORR of 32% while maintaining a good toxicity profile (14% G ≥ 3).

The safety of anti-CTLA-4 agents has also been addressed. Formentiet al[67] designed a phase I/II study with 39 patients treated with ipilimumab plus SABR (28.5 Gy in 3 fx or 30 Gy in 5 fx). ORR was 31%, PFS was 7.1 mo and OS was 13 mo, with only 10.3% G ≥ 3 toxicity[67]. The safety of multisite irradiation was evaluated in the oligometastatic setting by Bauml *et al*[68], who included 45 patients with oligometastatic NSCLC and delivered local ablative therapy (surgery or SABR) followed by sequential pembrolizumab, showing promising results in terms of PFS (19.1 mo) and OS (41.6 mo), with low toxicity rates.

With the safety of the combination established and the promising survival data reported in these initial studies, randomized evidence on the combination of ICI and RT (mainly in the form of SABR, also known as I-SABR) is finally starting to emerge. At the moment, three randomized trials have published their results. Not only are these reinforcing the idea that a benefit in survival exists, but they are also starting to contemplate some questions regarding the optimal treatment delivery. For instance, the COSINR study by Patel *et al*[69] is a phase I trial that randomized 35 patients to receive dual ICI (ipilimumab plus nivolumab) and either concurrent or sequential SABR. Global ORR was 68%, while PFS was 6.2 mo in the concomitant arm and 5.9 mo in the sequential arm. Welsh *et al*[70] recently reported the results of a phase II study that randomized 72 patients to receive RT plus pembrolizumab *vs* pembrolizumab monotherapy. In the experimental arm, patients received either SABR (50 Gy in 4 fx or 70 Gy in 10 fx) or conventional RT (45 Gy in 15 fx). Globally, there were no significant differences in response or PFS between the combination arm and the pembrolizumab arm, with an ORR of 22% *vs* 25 and PFS of 9.1 *vs* 5.1 (P = 1.00). However, in the subanalysis of patients treated in the combination arm, ORR was higher in the SABR group than in the conventional RT group (38% *vs* 10%), as well as PFS (20.8 mo *vs* 6.8 mo, P = 0.03)[70]. Finally, the PEMBRO-RT phase II study included 76 patients and randomized them in two arms: sequential pembrolizumab after SABR to a single lesion (24 Gy in 3 fx) *vs* pembrolizumab monotherapy. ORR was 36% and 18%, respectively. Furthermore, PFS favored the I-SABR arm (6.6 mo *vs* 1.9 mo), as well as OS (15.6 mo *vs* 7.6 mo), even though these were not statistically significant[71].

At present, a great number of clinical trials combining RT and ICI in stage IV NSCLC are ongoing (Table 5). These include multiple ICI agents (atezolizumab, avelumab, nivolumab, pembrolizumab, sintilimab, tremelimumab), different combinations, various treatment sequences (induction, sequential or concomitant) and several fractionations and RT techniques such as conventional RT, hypofractionation, SABR, intensity-modulated RT and proton beam RT.

CONCLUSION

Radio-immunotherapy represents a dynamic area of preclinical and clinical investigation in lung cancer. The synergy between RT and ICI to achieve a greater tumor response has been shown to be a promising option for the treatment of NSCLC. The positive experiences reported with the combination of RT and ICI in early stage, unresectable stage III and stage IV NSCLC have reinforced the interest in the association of these two treatments. In the years to come, the results of ongoing clinical trials will continue to evolve clinical practice in NSCLC.

Table 5 Ongoing clinical trials involving radiotherapy and immunotherapy in stage IV non-small cell lung cancer

Ref.	Phase	ICI agent	RT dose	Design	Primary endpoints
NCT03158883[93]	I	Avelumab	50 Gy/5 fx	ICI + SABR	ORR
NCT03224871[94]	I	Nivolumab; Pembrolizumab; Intratumor IL-2	8 Gy/3 fx	ICI + IL-2 + RT	MTD
NCT03436056, PRIMING [95]	I	Pembrolizumab	SABR 30 Gy-3 fx SABR 54 Gy/3 fx	ICI + SABR	MTD
NCT03812549[96]	I	Sintilimab	SABR 30 Gy/3 fx; LD (low dose)-RT: 2 Gy/1 fx or 4 Gy/2 fx or 10 Gy/5 fx	ICI + SABR; ICI + LD-RT	MTD
NCT03223155, COSINR [69]	I	Nivolumab; Ipilimumab	SABR 3-5 fx, 2-4 sites	ICI + SABR	MTD
NCT02639026[97]	I	Durvalumab; Tremelimumab	HFRT 24 Gy/3 fx, 17 Gy/1 fx	ICI + HFRT	MTD
NCT03275597[98]	I	Durvalumab; Tremelimumab	SABR 30-50 Gy/5 fx	ICI + SABR	MTD
NCT03168464[99]	I-II	Nivolumab; Ipilimumab	RT 30 Gy/5 fx	ICI + RT	ORR
NCT02239900[100]	I-IIR	Ipilimumab	SABR 50 Gy/4 fx or 60 Gy/10 fx; 1-4 lesions	ICI + SABR	MTD
NCT02444741[101]	I-IIR	Pembrolizumab	SABR 4 fx or IMRT, PBRT, 3D-CRT 15 fx	ICI + SABR or IMRT, PBRT, 3D-CRT	MTD, ORR
NCT03176173, RRADICAL [102]	II	Nivolumab; Pembrolizumab; Atezolizumab	SABR 1-10 fx	ICI +/- SABR	PFS
NCT03965468, CHESS[103]	II	Durvalumab	SABR 1-10 fx	ICI + SABR + CT	PFS
NCT03044626, FORCE [104]	II	Nivolumab	RT 20 Gy/5 fx	ICI + RT	ORR
NCT02221739[105]	II	Ipilimumab	IMRT or 3D-CRT 30 Gy/5 fx	ICI + RT	ORR
NCT02658097[106]	II	Pembrolizumab	RT 8 Gy/1 fx	ICI + RT	ORR
NCT03391869, LONESTAR [107]	III	Nivolumab; Ipilimumab	LCT	ICI +/- SABR	OS
NCT03867175[108]	III	Pembrolizumab	SABR 3-10 fx	ICI +/- SABR	PFS
NCT03774732, NIRVANA-LUNG[109]	III	Pembrolizumab	SABR or 3D-CRT 18 Gy/3 fx	ICI + RT + CT	OS

ICI: Immune checkpoint inhibitors; Fx: Fraction; SABR: Stereotactic ablative radiotherapy; RT: Radiotherapy; LD-RT: Low dose radiotherapy; HFRT: Hypofractionated radiotherapy; IMRT: Intensity modulated radiotherapy; PBRT: Proton beam radiation therapy; 3D-CRT: 3D conformal radiation therapy; LCT: Local consolidation therapy; CT: Chemotherapy; ORR: Overall response rate; MTD: Maximum tolerated dose.

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MINIREVIEWS

Consensus molecular subtypes of colorectal cancer in clinical practice: A translational approach

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Abstract

The identification of several genetic mutations in colorectal cancer (CRC) has allowed a better comprehension of the prognosis and response to different antineoplastic treatments. Recently, through a systematic process, consensus molecular subtypes (CMS) have been described to characterize genetic and molecular mutations in CRC patients. Through CMS, CRC patients can be categorized into four molecular subtypes of CRC by wide transcriptional genome analysis. CMS1 has microsatellite instability and mutations in CIMP and BRAF pathways. CMS2, distinguished by mutations in specific pathways linked to cellular metabolism, also has a better prognosis. CMS3 has a KRAS mutation as a hallmark. CMS4 presents mutations in fibrogenesis pathways and mesenchymal-epithelial transition, associated with a worse prognosis. CMS classification can be a meaningful step in providing possible answers to important issues in CRC, such as the use of adjuvant chemotherapy in stage II, personalized first-line chemotherapy for metastasic CRC, and possible new target treatments that address specific pathways in each molecular subtype. Understanding CMS is a crucial step in personalized medicine, although prospective clinical trials selecting patients by CMS are required to pass proof-of-concept before becoming a routine clinical tool in oncology routine care.

Key Words: Colorectal neoplasms; Precision medicine; Microsatellite instability; Next-generation sequencing

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Core Tip: Colorectal cancer has a variable response to different treatments that could be

quality classification
Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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explained by genetic and molecular heterogeneity in the neoplasm. Recently, a novel classification according to consensus molecular subtype has been proposed to explain this neoplasm heterogeneity. From a clinical oncology perspective, this classification opens opportunities to resolve some current clinical questions in the treatment of colorectal cancer.

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INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of death by cancer worldwide[1]. Despite important advances in early diagnosis and management, 25% of patients debut in metastatic stages and 50% localized stages, later presenting disseminated disease[2]. Currently, CRC management is based on tumor-node-metastasis (TNM) system staging, and in recent years, on several genetic mutations such as microsatellite instability (MSI), KRAS/NRAS, and BRAF. These mutations have a role in selecting better candidates for certain systemic therapies[3,4]. Improvements in classic systemic therapies for CRC have allowed more effective and tolerable chemotherapy regimens, mainly based on fluoropyrimidines with oxaliplatin and/or irinotecan. Proposing novel target therapies is also possible for selected patients[5,6].

A new paradigm has resulted from the problem of heterogeneity of CRC[7], which explains the significant impact of variable responses to classic systemic therapies. Thus, some patients present satisfactory and sustained responses. In contrast, other patients with CRC present low response rates to standard therapies, with rapidly progressive disease and high mortality. It has been argued that one way to approach this paradigm is through the characterization and creation of a framework based on genetic and molecular characteristics to explain the heterogeneity of colon cancer. Recently, a major initiative has emerged to describe CRC heterogeneity. The consensus molecular subtypes (CMS) provide a systematic way to classify CRC into four molecular subtypes according to their molecular and genetic profile[8].

Characterizing molecular subtypes in the CRC could optimize the management of these patients. Through knowledge of the biology of the disease, we could better predict the response to therapeutic alternatives to select the most appropriate therapy for each patient[9,10]. This approach is a crucial step in the development of personalized therapies in this disease. In this context, the current review aims to present a translational approach for routine oncology clinical practice regarding the implications of CMS classification with a focus on prognosis and promising novel antineoplastic agents in different stages of CRC.

CHARACTERISTICS OF EACH CMS

In recent years, different models have been proposed based on genetic, molecular, epigenetic, and phenotypic profiles to explain the heterogeneity of CRC[11-15], obtaining different molecular classifications with different clinical outcomes. A major collaborative effort to integrate all CRC classifications into a single model was identified by experts from the CRC Subtyping Consortium through the analysis of six independent classification studies, obtaining a CRC classification of four CMS[8]. This presents an integrated framework to capture the heterogeneity of CRC at the gene expression and molecular level through transcriptome-wide analysis[9]. The methodology of the consensus assessed redundant pathways and upregulation of signaling pathways that are independent of DNA mutations to provide a characterization of the molecular status[16].

CRC classification based on genome-wide transcriptional profiles has seen important research developments during the last decade; no single genetic defect can be unequivocally assigned to a specific molecular profile. CMS classifications have certain

hallmarks characterizing each subtype[9,17,18]. In brief, each CMS is characterized as follows.

CMS1 (immune) is characterized by MSI, with high mutations of CIMP and BRAF and a low prevalence of SNCA. It is associated with lymphocyte infiltration and immune activation, in addition to hypermethylation and decreased signaling through the WNT pathway[19].

CMS2 (canonical) has epithelial characteristics. It is characterized by high chromosomal instability, high somatic copy number alterations (SCNA) counts, and WNT and MYC mutations, causing high activity of these intracellular signaling pathways [18,19]. It also features increased expression of EGFR, its ligands AREG and EREG, and TP53, APC, and RAS mutation[20]. These can be distinguished from other CRC subtypes by marked upregulation of the downstream targets of WNT and MYC and increased expression of EGFR oncogenes, ERBB2 (also known as HER2), insulin-like growth factor 2, insulin receptor substrate 2, hepatocyte nuclear factor transcription factor 4, and cyclins[19].

CMS3 (metabolic) has a distinctive global genomic and epigenomic profile with mixed characteristics, metabolic reprogramming, and dysregulated pathways, with increased activity in glutaminolysis and lipidogenesis[20], enriched with KRAS-activating mutations. It presents a moderate or low mixed state of MSI and intermediate CIMP, and moderate activation of WNT and MYC, with PIK3CA mutation and IGBP3 overexpression, without BRAF mutations[19].

CMS4 (mesenchymal) has positive gene regulation and overexpression of proteins involved in stromal infiltration, mesenchymal activation, extracellular matrix remodeling, neoangiogenesis, prominent TGF-β activation, and complement pathways. These are characteristic of mesenchymal epithelial transition, overexpressing EMT genes, evidence of prominent EMT gene TGF-β activation, and high SCNA counts. Six immune genes (PROK1, THBS1, FGF11, CRP, S100A14, and CCL19) have been identified as the key factors of CMS4 and can potentially be applied for risk assessment of CRC patients[19,21]. The main hallmarks of CMS are briefly described in Figure 1.

IDENTIFYING THE PATIENT MOLECULAR SUBTYPE

Classification by CMS in real clinical settings, outside clinical trials, is challenging. In recent years, certain genetic hallmarks have been routinely determined. For example, MSI mutations are involved in advanced stages of CRC and are highly predictive of CMS1. MSI can be detected by polymerase chain reaction based on a panel of different microsatellite loci or applying immunohistochemistry (IHC) with antibodies against mismatch repair (MMR) proteins[22]. For the other subtypes, heterogeneous groups of genetic and molecular conditions are commonly found in pathogenic mutations in CRC, such as KRAS, BRAF, and APC[9,22]. For these mutations, in clinical settings, a commercial genetic panel that includes genes contained in CMS are the NanoString nCounter®, Almac Xcel microarray assay, and Affymetrix GeneChip® Human Transcriptome Array 2.0 (HTA).

Several research groups have aimed to obtain a practical and robust CMS classifier that works on formalin-fixed, paraffin-embedded primary CRC tissues, based on gene expression or IHC[23]. Categorizing patients relies fundamentally on mutational, transcriptomic, and proteomic data analysis[24]. A novel CMS classifier based on a filtered set of cancer cell-intrinsic, subtype-enriched gene expression markers, referred to as CMS caller[25], provides robust classification of CMS groups in datasets generated on different gene expression platforms and biological sample types, readily available for its purpose. A 40-gene ColoType signature has recently been developed, which uses genome-wide assays, frozen tissue-specific RNA sequences, or FFPE. The results correlate highly with those reported by the other two systems, in addition to allowing accurate and reproducible CMS subtype analysis for clinical applications[26].

Routinely practicing CMS classification for patients with CRC is challenging due to the difficult applicability and costs of this method[27]. However, an IHC approach has been proposed, which could represent a reasonable option for the molecular classification of CRC through morphological phenotype and a simpler way to guide case management[23,28]. Several IHC protocols have been proposed, such as a phenotypic subtyping method based on immune infiltrate, stromal invasion, and proliferation rate [27]. Another protocol proposed to correlate specifically with CMS is the IHC detection of MSI with antibodies against MSH1-2 MMR proteins, allocating samples with high-level MSI to CMS1, then classifying the remaining subtypes through staining for four

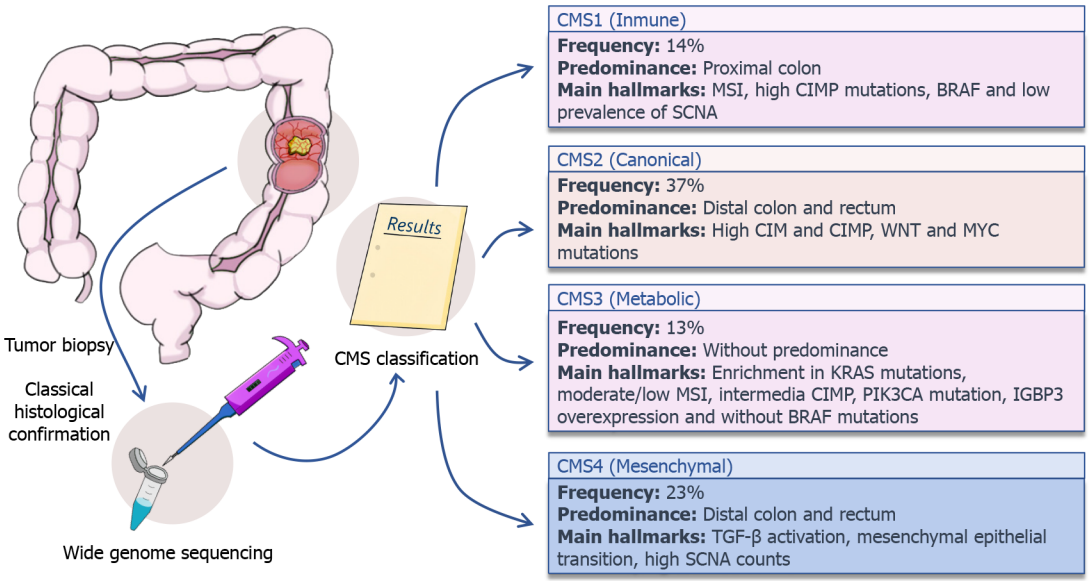


Figure 1 Main characteristics of consensus molecular subtypes in colorectal cancers. After the patient undergoes a biopsy, the diagnosis of colorectal cancer is made and subsequent stratification by tumor-node-metastasis is performed. Samples can be obtained to allow wide genome sequencing analysis with the objective of characterization into one of four consensus molecular subtypes. CMS: Consensus molecular subtypes; SCNA: Somatic copy number alterations.

gene product proteins (CDX2, FRD6, HTR2B, and ZEBI), allowing differentiation between CMS2/3 with CMS4[22]. This method, complemented with an IHC-based classifier, has demonstrated 87% concordance with transcriptome-based classification [23], indicating that IHC can be used to categorize CRC molecular subtypes, although not with 100% concordance. However, it has the disadvantage of being unable to distinguish between CMS2 and 3 because both subtypes share similar epithelial features. Recently, an improvement to this protocol has been proposed, adding IHQ for β-catenin to differentiate between CMS2 and 3, with 71.4% concordance compared to an RNA-sequencing-based classifier. This is based on CMS2 activating WNT pathway-regulated β-catenin expression[29].

However, the translation of CMS by genome-wide transcriptional profiles into clinical practice is subject to several obstacles[16], such as the complexity, difficulties of translation to routine pathology, and costs of this method of classification[24,27]. Until genomic profiling becomes more widespread in clinical practice, the molecular subtypes of CRC can be assessed by IHC but with less accuracy compared to the transcriptome gold standard[23]. Nevertheless, CMS is expected to be able to be used in routine clinical practice by overcoming these obstacles and becoming widely available in the near future for the classification of CRC through genome-wide transcriptional profiles.

CMS AND PROGNOSIS

Traditionally, the prognosis and treatment selection of patients with CRC has been based on the clinical pathological classification of TNM[30]. However, morphologically similar tumors can have different genetic expression and molecular profiles[9] with the capacity to determine different prognoses[31]. Currently, the main international clinical guidelines recommend determining certain biomarkers in specific clinical contexts, such as dMMR/MSI, BRAF, and RAS in metastatic CRC (mCRC) to select the optimal chemotherapy treatment[3,4]. In particular, the CMS classification was designed to categorize CRC heterogeneity in a transcriptional profile, although each subgroup has also been reported to have a different prognosis. In an analysis of 4151 patients with CRC[8], the overall survival (OS) in CMS4 was worse compared to CMS1–3, and a better OS was found in patients with CMS2. Notably, the OS calculation included patients in all TNM stages. In addition, CMS1 patients have a worse survival rate after relapse, and CMS2 patients have a longer survival after relapse.

Despite the important contribution of CMS classification to understanding the oncogenesis of CRC, whether this classification can better predict the prognosis of CRC patients is still uncertain[32]. In particular, in patients with stage II CRC, contro-

versies exist about the clinical benefits of chemotherapy[33]. Stage II patients are selected for chemotherapy if they have a pathological or clinical risk factor such as T4 status, suboptimal lymph node resection, perineural and perivascular invasion, or colon perforation[4]. In this context, it has been proposed that better markers such as CMS could provide a better selection of patients to undergo chemotherapy. Studies that shown that in stages II–III, patients with CMS 4 have a worse prognosis[34]. One study hypothesized that this is a consequence of resistance to fluorouracil–leucovorin regimens commonly used in these stages[35]. Likewise, in low-risk stage II CRC patients who did not undergo adjuvant chemotherapy, CMS4 had significantly worse outcomes in relapse-free survival (RFS) compared to other CMS groups[36]. Using IHQ CMS classification, Li *et al*[37] described a worse OS and disease-free survival in CMS4 and a better OS with adjuvant chemotherapy for stage II CRC in CMS2-3. Furthermore, it has been suggested that certain gene mutations in each CMS can modify outcomes. For instance, BRAF mutations were associated with metastasis in patients with MSS and CM1(OS 22% in BRAF mutated *vs* 81% in wild-type BRAF, P = 0.001). In CMS2-3 patients, mutated KRAS had worse outcomes (OS 59% in KRAS mutated *vs* 75% in wild-type KRAS)[38]. Moreover, patients with MSI and CMS1 have a better OS and RFS compared to CMS2-4. Contrary to previous results, Purcell *et al* [39] reported that stage II patients with CMS3 had a worse prognosis in OS than patients in CMS1-2, although an imbalance between CMS groups, with few CMS4 patients, could explain this result. Besides the possibility for CMS to determine CRC subtypes with worse prognosis for proposing personalized treatments, CMS still needs more studies to define the differences in the prognosis of patients through the different TNM stages of CRC.

In recent years, a special interest has emerged in defining molecular characteristics in patients with mCRC to select the best chemotherapy regimen[40]. In particular, the applicability of CMS has been studied most in this subgroup of patients. An analysis of the CALGB/SWOG 80405[16] trial determined the CMS of 664 patients using a genetic panel (NanoString). It found a positive relationship between OS and progression-free survival (PFS) and each CMS, determining a mean survival (months) of 15 for CMS1, 40.3 for CMS2, 24.3 for CMS3, and 31.4 for CM4, independent of assigned first-line chemotherapy treatment. In a sub-analysis of the AGITG MAX trial, 237 patients with mCRC were classified by CMS using an Almac Xcel microarray assay. A statistically significant association was found between CMS and OS, but not with PFS, independent of assigned first-line chemotherapy treatment. Similar OS were reported: CMS2 had the larger OS (median 24.2 mo), CMS1 had the worst (8.8 mo), and CMS3 (17.6 mo) and CMS4 (21.4 mo) had intermediate OS[41]. Similarly, the FIRE-3 trial[20] that included 438 patients categorized by CMS using the Almac Xcel microarray showed a correlation with OS and PFS independent of assigned treatment, with a worse mean OS in CMS1 (15.9 mo) and better OS in CM2 (29.0 mo). Whilst CM3 (18.6 mo) and CMS4 (24.8 mo) had a medium OS. Similar results were also reported by a retrospective analysis finding a worse OS in CMS1 and a better OS in CMS2[42]. Finally, a retrospective analysis of the TRIBE2 trial found better PFS and OS outcomes in CMS2 and CMS4 compared to CMS1 and CMS3[43]. A summary of the main prognoses in published reports of CMS is shown in Table 1. Questions remain in the prognosis of each CMS, especially when analyzing the results at each stage measured by TNM. However, encouraging results have been seen when predicting the subtypes of patients with worse prognoses in mCRC and stage II, opening possibilities to propose personalized treatments based on the molecular landscape of the CRC of each patient.

IMPORTANCE OF CONSIDERING CMS FOR FUTURE CLINICAL TRIALS

Considering the significant recent advances in the molecular and genetic profile of CRC through CMS classification, this knowledge must be projected using a proof-of-concept approach, applying it in clinical trials[17]. Patient selection by CMS characterization could be a crucial step in cancer staging and personalized treatment guided by biomarker selection. However, CMS interpretation in the context of clinical trials has some factors that need to be considered when interpreting the results, such as the sample collection site (colon *vs*. rectum), the trial inclusion and exclusion criteria, the first-line chemotherapy scheme used, specific mutations that alone produce different outcomes, and the method used to predict the CMS[44]. Despite these limitations, the identification of CMS in future clinical trials is projected to allow better precision in selecting specific treatments for each patient, especially in the use of immunotherapy

Table 1 Outcomes in four consensus molecular subtype profiles

Ref.	*n*	Outcomes	CMS1, mean (95%CI)	CMS2, mean (95%CI)	CMS3, mean (95%CI)	CMS4, mean (95%CI)
Stages I-IV						
Purcell *et al*[39], 2019	257	5-yr OS (%)	63.7 (51.1-79.4)	64.4 (56.6-73.4)	52.8 (37.1-75.1)	42.8 (23.8-76.8)
		5-yr PFS (%)	61.2 (48.8-76.8)	59.8 (51.8-68.9)	52.7 (47.5-74.0)	38.8 (21.0-71.9)
Guinney *et al*[8], 2015	2129	5-yr OS (%)	74 (70-75)	77 (74-80)	75 (70-80)	62 (58-66)
		5-yr DFS (%)	75 (70-80)	73 (70-77)	73 (68-80)	60 (55-65)
Stage II						
Shinto *et al*[36], 2020	232	5-yr DFS (%)	100	85.5	92.3	73
Stage IV						
Borelli *et al*[43], 2021	426	OS (mo)	13.7 (6.1-27.9)	27.0 (23.9-30.1)	18.3 (16.1-24.0)	26.2 (21.4-29.9)
		PFS (mo)	5.4 (3.8-9.9)	12.9 (11.0-14.3)	8.3 (7.4-10.1)	10.7 (9.8-13.1)
Stintzing *et al*[20], 2019	438	OS (mo)	15.9 (11.0-20.8)	29.0 (26.7-31.4)	18.6 (15.4-21.7)	24.8 (22.6-27.1)
		PFS (mo)	8.2 (6.7-9.6)	11.7 (10.8-12.6)	8.5 (6.8-10.3)	9.6 (8.6-10.6)
Lenz *et al*[16], 2019	581	OS (mo)	15.0 (11.7-22.4)	40.3 (36.1-43.1)	24.3 (16.4-29.0)	31.4 (26.3-36.9)
		PFS (mo)	7.1 (5.7-8.6)	13.4 (12.8-15.4)	8.7 (7.2-9.8)	11.0 (9.7-12.0)
Mooi *et al*[41], 2018	237	OS (mo)	8.8 (6.5-16.0)	24.2 (19.1-27.4)	17.6 (11.3-24.6)	21.4 (15.8-23.1)
		PFS (mo)	No statistical differences in this cohort			
Okita *et al*[42], 2018	193	OS (mo)	21.4 (13.3-35.5)	48.1 (34.8-65.6)	38.7 (30.6-45.6)	44.0 (33.0-50.5)

OS: Overall survival; DFS: Disease-free survival; PFS: Progression-free survival.

for mCRC[10,45]. In the case of immunotherapy, ongoing trials (NCT03436563) are testing M7824 treatment, an anti-PD-L1/TGF-β Trap fusion protein. This treatment has demonstrated an anti-tumor response by TGF-βand PD-L1 immunosuppressive pathways with successful results in murine CRC models[46,47]. It has been proposed as a possible treatment for CMS4 because it activates the TGF-pathway[9,48]. In addition, CMS identification could allow selecting a personalized first-line chemotherapy regimen in mCRC. For instance, a possible hypothesis has recently been proposed after analysis through CMS classification in two important clinical trials, FIRE-3[20] and CALGB/SWOG 80405[16], which both compared the response to first-line chemotherapy in addition to cetuximab or bevacizumab. The authors theorized that the combination of irinotecan and cetuximab in all CMS classification, when patients have received oxaliplatin, has a synergic effect in CMS2 and CMS3, but in CMS1 and CMS4 it has an antagonistic effect due to the poor efficacy of oxaliplatin in a fibroblast-rich microenvironment[49]. Recently, different retrospective studies of clinical trials have shown associations between CMS and the prognosis of different chemotherapy regimens[43,50]. However, these results must be confirmed using clinical trials with prospective designs that include different CMS patients.

CONCLUSION

The CMS provides an interesting opportunity to explore the heterogeneity of CRC. CMS classification can approximate research in frequently unsolved daily clinical practice problems. For instance, in patients with stage II colon cancer, where the benefit of chemotherapy is still unclear, CMS classification could determine which patients would benefit from adjuvant chemotherapy. Likewise, CMS will allow defining the best first-line chemotherapy regimen in mCRC. Understanding the genetic profile of tumors could allow developing new interventions to target treatments that address specific pathways to each molecular subtype. Therefore, CMS comprehension is a crucial step towards personalized medicine, though any interesting perspective must be proven through prospective clinical trials selecting patients by CMS.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Real-world evaluation of upfront docetaxel in metastatic castration-sensitive prostate cancer

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Informed consent statement: Based on the retrospective and

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Abstract

BACKGROUND

The majority of patients with newly diagnosed metastatic prostate cancer (PC) initially respond to androgen deprivation therapy (ADT) and are classified as metastatic castration-sensitive PC (mCSPC). Following months to years of ADT, the disease tends to become resistant to ADT. Recent randomized phase-III trials demonstrated a survival benefit with the addition of upfront docetaxel to ADT in mCSPC. Following its implementation in routine care, this combined treatment strategy requires more detailed evaluation in a real-world setting.

AIM

To assess the real-world outcome and safety of upfront docetaxel treatment in mCSPC.

METHODS

A multicenter retrospective cohort study in the Southeast Health Care Region of Sweden was performed. This region includes approximately 1.1 million citizens and the oncology departments of Linköping, Jönköping, and Kalmar. All patients

noninterventional nature of the study and the absence of publication of individual data, the ethics board did not consider it possible or necessary to obtain written informed consent.

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given upfront docetaxel for mCSPC from July 2015 until December 2017 were included. The primary endpoint was progression-free survival (PFS) at 12 mo, and the secondary endpoints were PFS at 24 mo, overall survival (OS), treatment intensity, adverse events, and unplanned hospitalizations. Exploratory analyses on potential prognostic parameters were performed.

RESULTS

Ninety-four patients were eligible and formed the study cohort. PFS at 12 and 24 mo was 75% (95%CI: 66–84) and 58% (46–70), respectively. OS at 12 and 24 mo was 93% (87–99) and 86% (76–96). A total of 91% of patients (n = 86) were given docetaxel according to the standard protocol of 75 mg/m^2 every 3 wk (6 cycles), while 9% (n = 8) received a modified protocol of 50 mg/m^2 every 2 wk (9 cycles). The average overall dose intensity for those commencing standard treatment was 91%. Univariate Cox regression analyses show that baseline PSA > 180 *vs* < 180 and the presence of distant metastases *vs* locoregional lymph node metastases were only negative prognostic factors (HR 2.86, 95%CI: 1.39–5.87, P = 0.0041 and 3.36, 95%CI: 1.03–10.96, P = 0.045). Following multivariate analysis, statistical significance remained for PSA (2.51, 95%CI: 1.21–5.19, P = 0.013) but not for metastatic status (2.60, 95%CI: 0.78–8.65, P = 0.12). Febrile neutropenia was recorded in 21% (n = 20) of patients, and 26% (n = 24) had at least one episode of unplanned hospitalization under and up to 30 d after the treatment course.

CONCLUSION

Results from this study support the implementation of upfront docetaxel plus ADT as part of the standard of care treatment strategy in mCSPC.

Key Words: Prostate cancer; Chemotherapy; Docetaxel; Castration sensitive; Metastatic; Real world

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Core Tip: Two recent trials reported impressive outcomes when upfront docetaxel is added to androgen deprivation therapy in metastatic castration-sensitive prostate cancer (mCSPC). This study presents the outcome and safety of this treatment strategy in a real-world context of all eligible patients in the southeast region of Sweden. While the treatment is toxic in terms of febrile neutropenia and unplanned hospitalizations, the outcome and long-term prognosis appear similar in real life and randomized controlled trial contexts. Further implementation of upfront docetaxel in mCSPC in routine care is encouraged.

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INTRODUCTION

Prostate cancer (PC) is the second most common malignancy in men. In 2018, more than 1.2 million new cases were reported worldwide, which corresponds to approximately 7% of all cancers[1]. In Sweden, the annual incidence is approximately 10500, with a median age of onset of 68 years[2]. While the 5-year overall survival (OS) (for all stages combined) is continuously improving and now exceeds 90%, the prognosis for patients presenting with upfront metastases remains less optimistic, with expected OS in the range of 30–36 mo and 5-year survival of approximately 30%[3-9].

The majority of patients with newly diagnosed metastatic PC will initially respond to androgen deprivation therapy (ADT) and are classified as castration sensitive (mCSPC). Following months to years of ADT, the disease will tend to become resistant to ADT and thus be defined as metastatic castration refractory prostate cancer

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(mCRPC). In mCRPC, palliative chemotherapy with docetaxel may offer temporary relief, but survival benefits are usually limited to a few months[4]. However, two recent multicenter trials demonstrated a considerable benefit for docetaxel when this drug was introduced early (*i.e.*, in the initial castration-sensitive phase of the disease) [10,11].

In the CHAARTED study, 790 men were randomized into six cycles of docetaxel plus ADT *vs* ADT alone. Patients were stratified according to high (visceral metastases or ≥ 4 bone lesions with ≥ 1 beyond the vertebral bodies and pelvis) or low-volume disease. The median OS was 58 mo for men treated with ADT plus docetaxel and 44 mo for men treated with ADT alone. In men with high-volume disease, the additive effect of docetaxel was even better, with a median OS benefit of 17 mo (49 mo *vs* 32 mo)[11].

STAMPEDE was a multiarmed, multistage trial that included 2962 men with both metastatic and nonmetastatic PC. Stratified randomization (2:1:1:1) allocated men to a standard of care (SOC): ADT with or without radiotherapy, SOC plus docetaxel, SOC plus zoledronic acid, and SOC plus docetaxel plus zoledronic acid. Sixty-one percent had distant metastasis, and 15% had node-positive disease. The remaining 24% presented with nonmetastatic high-risk locally advanced disease [T3/4, PSA ≥ 40 ng/mL, and/or Gleason score (GS) 8–10]. In the STAMPEDE trial, median OS was improved by 10 mo for SOC plus docetaxel compared with SOC alone (81 mo *vs* 71 mo). For the group with metastatic disease, the OS benefit was 15 mo for SOC plus docetaxel *vs* SOC alone (60 mo *vs* 45 mo)[10,12,13].

A smaller French study, GETUG-AFU 15, including approximately 400 patients who were randomized to receive ADT alone or ADT plus docetaxel[14], could not confirm the findings of CHAARTED and STAMPEDE. The French study did not reveal any statistically significant survival benefit with upfront docetaxel treatment (median OS 59 mo in the ADT plus docetaxel group *vs* 54 mo in the ADT alone group). The different outcomes of the various trials may depend on discrepancies in study populations. In the STAMPEDE and CHAARTED trials, the median PSA levels were nearly twice as high as the median PSA reported in the GETUG-15 population, indicating that the disease stage was generally more advanced in the former cohorts than in the latter. While 66% of CHAARTED patients were reported to have a high volume of metastases, only 48% were classified as such in GETUG-15. Differences were also observed in the GS, with a GS ≥ 8 reported for nearly 61% of the population in CHAARTED and 74% in STAMPEDE compared to 55% in GETUG-15[10,11,14]. Together, these findings suggest that the CHAARTED and STAMPEDE trials included patients with worse prognosis than the subjects enrolled in the GETUG-15 study.

Based on the promising results of STAMPEDE and CHAARTED, the addition of docetaxel to ADT in early mCSPC was introduced in international and national (including Swedish) guidelines, particularly for patients with high-volume disease, in 2015-2016. To be eligible for this therapy, patients should be in good general condition and without significant comorbidities[15]. The eligibility conditions in the Swedish national guidelines were used from the reported characteristics of the STAMPEDE and CHAARTED populations.

Since its introduction in routine care, it remains largely unknown to what extent the outcomes observed in the STAMPEDE and CHAARTED trials are evident in patients treated outside the frame of a randomized controlled trial. This study was therefore designed to assess the real-world outcome and safety of early docetaxel treatment for patients with mCSPC. To completely describe the real-world situation with patients of all ages and with or without concomitant comorbidities, all consecutive patients who received this treatment in the Southeast Health Care Region of Sweden since 2015 were included.

MATERIALS AND METHODS

A retrospective multicenter cohort study of all men diagnosed with primary mCSPC in the Southeast Health Care Region of Sweden was designed. This region covers approximately 1.1 million citizens and includes the oncology departments of Linköping, Jönköping, and Kalmar. These three centers provide all oncological treatments in the region. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Regional Ethics Review board in Linköping, Region Östergötland, Sweden (diary number 2018/139–31). Based on the retrospective and noninterventional nature of the study and the absence of publication of individual data, the ethics board did not consider it possible or necessary to obtain written

informed consent.

Inclusion criteria were as follows: Male sex, age 18 years or older, evidence of newly diagnosed mCSPC between July 2015 and December 2017 (ICD-10 code C61.x), defined as either node positive (N+) and/or distant metastatic (M+) disease, and administration of at least one cycle of upfront docetaxel chemotherapy in addition to ADT. ADT was initiated before the start of docetaxel, either in conjunction with the diagnosis of mCSPC or earlier (*i.e.*, for patients already receiving ADT in an earlier nonmetastatic disease setting). The exclusion criteria were recurrent disease, castration refractory disease, and patient refusal to undergo ADT. Otherwise, to completely describe the real world situation, no exclusion criteria were applied.

The Swedish Cancer Registry (SCR) was used to identify eligible patients (https://www.socialstyrelsen.se/statistik-och-data/register/alla-register/cancerregistret/). Reporting to the SCR is mandatory, and the registry achieves over 95% coverage for all malignant tumors. The CSAM Cytodos software system (CSAM Health AS, Oslo, Norway), a software program being used for the prescription and administration of chemotherapy at all participating centers, was used to identify those treated with docetaxel.

Medical records were reviewed, and data were registered in a standardized case report form where patient and tumor characteristics, baseline biochemistry, Eastern Cooperative Oncology Group (ECOG) performance status, treatment regimens, toxicity parameters, PSA levels, relapse, and vital status were recorded. Patient and tumor characteristics were recorded according to the TMN classification (8th edition by Union for International Cancer Control 2017), GS according to International Society of Urological Pathology 2014, and histology according to WHO 2004.

The patients included had received docetaxel according to any of two different regimens, either by intravenous infusion every three weeks at doses of 75 mg/m^2 for a total of six cycles according to the CHAARTED and STAMPEDE treatment protocols or at a dose of 50 mg/m^2 in two-week cycles for a total of nine cycles according to local guidelines. The latter was used by one site for patients who, for any reason and at the treating oncologist's discretion, were deemed unfit to receive the 75 mg/m^2 regimen.

Bone marrow toxicity was evaluated by blood cell counts in addition to other standard biochemical parameters prior to each dose. In general, treatment response was evaluated with PSA levels at days 18–20 in every cycle and, for most patients, with X-ray computed tomography at the end of the sixth cycle (or at the ninth cycle of the 50 mg/m^2 two-week cycles).

The primary endpoint was progression-free survival (PFS) at 12 mo. PFS was defined as the time to biochemical progression in accordance with the CHAARTED protocol, where a serologic increase of the PSA level of more than 50% above nadir, reached after initiation of ADT and with two consecutive increases at least two weeks apart, clinical progression due to increasing symptoms, or deterioration or disease progression according to RECIST 1.0 were considered progression. If the PSA nadir was less than 2 μg/L, a minimum increase of more than 2 μg/L was required[10,11]. In addition, an alternative definition of progression according to the Swedish national guidelines, which stipulate serologic increase ≥ 25% from lowest PSA value after start of latest given treatment (and a minimum absolute increase of ≥ 2 μg/L), worsening of clinical symptoms, or radiological disease progression according to RECIST 1.0, was similarly applied[15].

Secondary endpoints were PFS at 24 mo, OS and treatment intensity included dose reductions, premature termination and protocol modifications, and safety of docetaxel treatment in terms of registered bone marrow toxicity and unplanned hospitalizations under and within 30 d after the last docetaxel treatment cycle. Patients were followed until death or May 18, 2018, whichever occurred first.

Statistical analysis

All statistical analyses were performed in the per-protocol population, defined as all patients who received at least one dose of docetaxel. Patient characteristics and tumor and treatment data were reported as numbers and percentages for categorical variables and medians and ranges for continuous variables. PFS at 12 and 24 mo was estimated according to the CHAARTED[11] and Swedish national guidelines[15] definitions of progressive disease (PD), respectively. Median PFS and OS for the entire cohort and subgroups defined by age over and under median (68); PSA over and under median (180); comorbidities; and presence of distant metastases or locoregional lymph node metastases only were estimated using Kaplan–Meier survival analyses, and the significance of the differences in estimates of median survival was calculated using log rank test. Cox regression analysis with a 95% confidence interval was used to evaluate hazard ratios for the same subgroups. P values below 0.05 were considered

statistically significant. Analyses were performed using IBM SPSS statistics software (IBM, version 25). Any missing data are reported in the respective Table.

RESULTS

Patients and baseline characteristics

A total of 94 eligible patients with primary mCSPC treated with docetaxel and ADT were identified and included. Baseline characteristics are shown in Table 1. For comparison, published data from CHAARTED[11] and STAMPEDE[10] are also shown in Table 1. Median age was 68 years (range 49–79). Comorbidities were present in 53% (n = 50) of patients, of which hypertension was the most prevalent. Regarding ECOG PS, data were only available in 64 (70%) of the cases, of which an overwhelming majority were ECOG PS 0–1 (n = 61, 95%). Median PSA at diagnosis was 180 (range 2–7367). Clinical TNM staging was recorded in 84 (90%) of the subjects, with T3N1M1 being the most common staging. Of those with distant metastases, bone metastases were most prevalent (n = 74, 79%), followed by lymph node metastases (n = 54, 57%). Most tumors (n = 60, 64%) were classified as GS 8–10.

Treatment data

Eighty-two (87%) of the patients received a combination of gonadotropin releasing hormone (GnRH) and a nonsteroidal antiandrogen as the castration method, nine (10%) were treated with GnRH alone, and three (3%) were surgically orchidectomized. The median time from the start of ADT until the start of docetaxel was 63 d (range 8–400). The median duration of ADT was 331 d (range 5–1038) (Table 2). Seventy-seven patients (82%) received docetaxel according to the 75 mg/m^2 every six weeks schedule, and eight (8%) received docetaxel according to the modified schedule of 50 mg/m^2 every two weeks.

Of those 77 patients commencing the 75 mg/m^2 regimen, 63 (81%) completed all 6 cycles. The mean administered dose (of 75 mg/m^2 full dose) was 91%, corresponding to 139 mg docetaxel (Table 2).

Of those eight patients commencing the 50 mg/m^2 schedule, four (50%) completed all 9 planned cycles. For this regimen, the mean administered dose was 83% of the full dose, corresponding to 86 mg of docetaxel.

Nine patients started with the 75 mg/m^2 schedule but switched to the 50 mg/m^2 regimen during treatment. In this subgroup of patients, all nine fulfilled the expected 6 cycles.

In total, 33 (35%) underwent at least one dose reduction, 13 (14%) had a dose escalation, and 47 (50%) received the initially prescribed dose throughout the treatment period (Table 2).

Progression free and OS

PFS and OS in the entire cohort are shown in Figures 1 and 2. PFS at 12 mo in the total cohort of 94 patients was 75% (95%CI: 66%-84%) or 71% (95%CI: 61%-81%), depending on whether the definition of CHAARTED/STAMPEDE or the Swedish national guidelines was used. The corresponding proportions at 24 mo were 58% (95%CI: 46%-70%) and 55% (95%CI: 43%-67%) (Table 2). The OS rates at 12 and 24 mo were 93% (95%CI: 87%-99%) and 86% (95%CI: 76%-96%), respectively. Median PFS and median OS were not reached by the data cutoff date. At the time of analysis, 65 patients had evidence of disease (69%), and 14 had died (15%) (Table 1). The best response at the end of docetaxel treatment was complete response for six subjects (6%), partial response (n = 50, 53%), stable disease (n = 15, 16%), and PD (n = 11, 12%). Twelve (13%) of the patients were non-evaluable (NE) for PFS (Table 2). Median follow-up was 20 mo.

Univariate and multivariate regression analyses of progression-free and OS in subgroups

Cox regression analyses were performed to compare PFS and OS in the following subgroups: Age older than 68 years *vs* 68 years or younger, PSA higher than 180 μg/L *vs* less than 180 μg/L, comorbidities (yes/no) and absence of distant metastases vs. presence of any distant metastases. For continuous variables (age and PSA), patients were dichotomized according to their below or above the median value of the respective parameter. Univariate Cox regression analyses showed that baseline PSA higher than 180 and the presence of distant metastases were negative prognostic

Table 1 Baseline characteristics of the first 94 patients with metastatic castration-sensitive prostate cancer treated with docetaxel and androgen deprivation therapy between July 2015 and December 2017 in the Southeast Health Care region of Sweden

	Total, *n* = 94 (%)	CHAARTED, ADT + Docetaxel	STAMPEDE, Standard of care + Docetaxel
Age, yr			
Median	68.0	64	65
Range	49-79	36-88	40-81
Prostate-specific antigen (μg/L), at diagnosis			
Median	180	50.9	70
Range	2-7367	0.2-8540.1	1-9999
Comorbidities, *n* (%)	50 (53)		
Diabetes mellitus I and II	16 (17)		56 (9)
Hyperlipidemia	23 (24)		208 (35)
Hypertension	38 (40)		
Previous malignant disease[1]	11 (11)		
Performance status (ECOG)[2], *n* (%)			
0	46 (72)	277 (69.8)	
1	15 (23)	114 (28.7)	
2	3 (5)	6 (1.5)	
T category at diagnosis[3], *n* (%)			
T1	6 (6)		0
T2	17 (18)		60 (10)
T3	46 (49)		390 (66)
T4	11 (12)		105 (18)
TX	4 (4)		35 (6)
Not assessed	10 (11)		
N category at diagnosis[3], *n* (%)			
N0	29 (31)		260 (44)
N1	42 (45)		298 (50)
NX	23 (24)		34 (6)
Metastases[3], *n* (%)			
Non-distant metastasis[4]	19 (20)		
Distant metastases	75 (80)		362 (61)
Location, *n* (%)			
Bone metastases	74 (79)		307 (52)
Liver metastases	2 (2)		6 (1)
Lung metastases	12 (13)		13 (2)
Lymph node metastases	54 (57)		102 (17)
Gleason sum score, *n* (%)			
≤ 6	2 (2)	21 (5.3)	≤ 7
7	27 (29)	96 (24.2)	110 (19%)
8-10	60 (64)	241 (60.7)	436 (74%)
Unknown	5 (5)	39 (9.8)	46 (8%)

Histology (WHO 2004), *n* (%)	
Acinar adenocarcinoma	86 (92)
Ductal carcinoma	1 (1)
Mixed type	2 (2)
Unknown	5 (5)
Follow-up, months	20
Median (IQR)	13-28
Status last follow-up, *n* (%)	
Alive, no disease progression	15 (16)
Alive, disease progression	65 (69)
Dead of disease	14 (15)

[1]Previous non-prostate cancer: Not specified (*n* = 6), basal cell carcinoma (*n* = 1), malignant melanoma (*n* = 2), pancreatic cancer (*n* = 1), thyroid cancer (*n* = 1).
[2]ECOG: Eastern Cooperative Oncology Group performance status. This information was available for 64 of 94 patients (68%).
[3]Staging according to TNM classification (8th edition UICC 2017); clinical staging *n* = 85, pathological staging *n* = 7. No information was found for *n* = 2. X means that a substage was not defined.
[4]Locoregional lymph node metastases are referred to as non-distant metastases. Corresponding data from the CHAARTED and STAMPEDE randomized trials[10,11] are shown for comparison.
ADT: Androgen deprivation therapy; PFS: Progression-free survival.

factors (HR 2.86, 95%CI: 1.39–5.87, P = 0.0041 and 3.36, 95%CI: 1.03–10.96, P = 0.045). Following multivariate analysis, statistical significance remained for PSA (2.51, 95%CI: 1.21–5.19, P = 0.013) but not for metastatic status (2.60, 95%CI: 0.78–8.65, P = 0.12) (Table 3). Similar and statistically significant findings on baseline PSA and PFS were evident when the Swedish national guidelines criteria for progressive disease were used (Table 4).

Safety

Table 5 shows registered side effects and bone marrow toxicity according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE version 4). Most strikingly, 20 (21%) of the patients experienced febrile neutropenia, and 24 (26%) had at least one episode of unplanned hospitalization under and up to 30 d after the docetaxel treatment course. Other reported adverse events, as well as reasons for early termination of the treatment, are shown in detail in Table 5.

DISCUSSION

This population-based study explored the outcome and safety of combined upfront docetaxel and ADT in mCSPC in a real-world cohort including the first 94 patients undergoing this treatment strategy in the Southeast Health Care Region of Sweden, reporting outcome and safety measures that were similar to previous findings in pivotal randomized controlled trials on the topic[10,11].

In the last few years, treatment of mCSPC with upfront docetaxel in addition to ADT has been widely implemented in routine care in Sweden and elsewhere. This significant change in practice was primarily based on the results of two major randomized controlled trials, CHAARTED and STAMPEDE[10,11]. While the results of these trials were promising, less is known about the outcome and safety of this treatment in a real-world context (*i.e.*, among patients who are treated outside the frame of a randomized controlled trial).

Based on the CHAARTED/STAMPEDE definition of progressive disease, the cohort investigated in this study exhibited PFS rates of 75% and 58% at 12 and 24 mo, respectively, closely corresponding to the outcomes displayed in the CHAARTED and STAMPEDE publications[10,11]. Similarly, OS estimates at 12 and 24 mo of 93% and 86% mirror the Kaplan–Meier curves of the two trial populations. Taken together, these results indicate that the value of upfront docetaxel added to ADT in mCSPC appears similar in randomized controlled populations and this Swedish real-world cohort.

Table 2 Treatment data

	Total, *n* = 94 (%)
ADT	
GnRH and nonsteroidal antiandrogen	82 (87)
GnRH alone	9 (10)
Orchidectomy	3 (3)
ADT	
Time from ADT start to Docetaxel start, days	
Median (range)	63 (8-400)
ADT duration, days	
Median (range)	331 (5-1038)
Docetaxel	
75[1] mg/m²	77
Adm mean dose % of full dose	91
Mean adm dose, mg	139
Mean acc dose, mg	758
Completed all cycles	63 (67)
50[2] mg/m²	8
Adm mean dose % of full dose	83
Mean adm dose, mg	86
Mean acc dose, mg	610
Completed all cycles	4 (50)
Switch	9
Adm mean dose % of full dose	87
Mean adm dose, mg	107
Mean acc dose, mg	641
Completed all cycles	9 (100)
Dose reduction	33 (35)
Dose escalation	13 (14)
Unchanged	47 (50)
Missing	1 (1)
Best response at end of Docetaxel[3]	
CR	6 (6)
PR	50 (53)
SD	15 (16)
PD	11 (12)
NE	12 (13)
Est. PFS	**Mean (95%CI)**
12 mo	
CHAARTED/STAMPEDE	75% (66-84)
Swedish national guidelines	71% (61-81)
24 mo	
CHAARTED/STAMPEDE	58% (46-70)

Swedish national guidelines	55% (43-67)
OS	
12 mo	93% (87-99)
24 mo	86% (76-96)

[1]75 mg/m^2 is given in 6 cycles every 21 d.
[2]50 mg/m^2 is given in 9 cycles every 14 d.
[3]CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease. (PD definition CHAARTED: Serologic increase of PSA level of more than 50% above nadir reached after initiation of androgen deprivation therapy, two consecutive increases at least 2 wk apart. If the nadir was less than 2 μg/L, a minimum increase of more than 2 μg/L was required. STAMPEDE: Biochemical progression rose by 50% above the within 24-wk nadir and above 4 μg/L. Radiologic according to RECIST version 1.0 Swedish national guidelines: Clinical: Increasing symptoms or deterioration of general condition. Serologic ≥ 25 percent from lowest PSA value after start of latest given treatment an augmentation of at least ≥ 2 μg/L is required. Radiologic progression of existing or new metastasis).
NE: Nonevaluable; ADT: Androgen deprivation therapy; PFS: Progression-free survival.

Table 3 Progression-free survival (according to the CHAARTED and STAMPEDE definitions of progressive disease) age, PSA, comorbidities and bone metastases

	Number of patients	Number of events	HR, 95%CI, *P* value (univariate)	HR, 95%CI, *P* value (multivariate)
Age, yr (median)				
≤ 68	48	21	1.00	1.00
> 68	46	15	0.78, 0.40-1.51, 0.45	0.83, 0.42-1.67, 0.61
PSA (median)				
≤ 180	48	12	1.00	1.00
> 180	46	24	2.86, 1.39-5.87, 0.0041	2.51, 1.21-5.19, 0.013
Comorbidities				
No	44	15	1.00	1.00
Yes	50	21	1.15, 0.59-2.23, 0.68	1.19, 0.60-2.36, 0.63
Distant metastases				
No	19	3	1.00	1.00
Yes	75	33	3.36, 1.03-10.96, 0.045	2.60, 0.78-8.65, 0.12

There are some similarities and differences between the real-world population investigated in this study and the patient populations of CHAARTED and STAMPEDE. In this cohort, patients were marginally older with a median of 68 years compared to a median of 64 (CHAARTED) and 65 (STAMPEDE) in the randomized controlled trials. The vast majority of patients (95%–99%) in the real-world cohort as well as the phase-III trial populations exhibited ECOG PS 0–1. Conversely, baseline PSA levels were considerably higher in the real-world cohort (median 180) than in the controlled trial populations, which exhibited median PSA levels of 51 (CHAARTED) and 70 (STAMPEDE), potentially indicating a higher disease burden in the real-world cohort at the start of the treatment. Patients with PSA above the median also had a significantly higher risk of progressive disease, which was reflected in both univariate and multivariable regression analyses (Figure 2).

Also, the extent of metastatic disease was different in the two phase-III trials and this real-world cohort. The STAMPEDE trial reported that 61% of the total study population had metastatic disease, and the CHAARTED trial, which only included patients with evidence of metastatic disease, reported 65% with high volume disease; these latter numbers were not available from the STAMPEDE publication. In this real-world cohort, 80% had distant metastases, while 20% had non-distant metastases only. While metastatic burden was a negative prognostic factor in univariate regression analysis of this cohort, the statistical significance did not remain in multivariable analysis.

Taken together, these findings indicate that the outcome of docetaxel in mCSPC is comparable in real life, where patients are generally older and often present with more

Table 4 Progression-free survival (according to Swedish national guidelines) age, PSA, comorbidities and bone metastases

	Number of patients	Number of events	HR, 95%CI, *P* value (univariate)	HR, 95%CI, *P* value (multivariate)
Age, yr (median)				
≤ 68	48	22	1.00	1.00
> 68	46	17	0.83, 0.44-1.56, 0.55	0.88, 0.46-1.71, 0.71
PSA (median)				
≤ 180	48	13	1.00	1.00
> 180	46	26	2.86, 1.44-5.69, 0.0028	2.57, 1.28-5.16, 0.0081
Comorbidities				
No	44	17	1.00	1.00
Yes	50	22	1.06, 0.56-2.00, 0.85	1.08, 0.56-2.07, 0.83
Distant metastases				
No	19	4	1.00	1.00
Yes	75	35	2.69, 0.96-7.59, 0.061	2.11, 0.73-6.06, 0.17

advanced disease in terms of baseline PSA levels, and phase-III trial populations with more beneficial baseline characteristics.

Completion of all planned cycles was reported in 86% of the CHAARTED and 76% of the STAMPEDE trial populations. Similar figures were found in this cohort: 81% completed the entire treatment course, and 35% (n = 33) underwent dose reductions. Eight (8%) also received a modified 50 mg/m^2 every-two-weeks schedule from the start, and nine (10%) converted from the standard 75 mg/m^2 every-three-weeks to this modified 50 mg/m^2 protocol (switch). There is currently little evidence for this 50 mg/m^2 protocol in mCSPC, and the deviation from SOC probably reflects an eagerness to provide the treatment to frail and/or comorbid patients who otherwise would be considered not eligible for docetaxel. The low number of patients in this subgroup together with the finding that only 50% of the patients who began with 50 mg/m^2 were able to fulfil all planned cycles mean that the efficacy and safety for this adapted treatment schedule remain unproven.

Safety data of this study reveal that 21% of the patients experienced febrile neutropenia and, in total, 26% had at least one episode of unplanned hospitalization under or shortly after the docetaxel treatment course. While only 4% had their treatments prematurely terminated due to febrile neutropenia, these findings still emphasize that the docetaxel 75 mg/m^2 every-three-weeks regimen is a particularly toxic treatment. This result might be particularly important when upfront docetaxel is considered for older and/or frail patients who would not be eligible for the STAMPEDE and CHAARTED trials.

To our knowledge, this is the first study that systematically reports the real-world outcome of upfront docetaxel in a Scandinavian context. Other real-world studies conducted in other countries and/or ethnic groups corroborate the results of this study. Lavoie *et al*[16] assessed the clinical effectiveness of upfront docetaxel in a Canadian setting, showing a similar outcome and safety data to those of this study, with 12-mo OS of 91% and 26% experiencing grade 3–4 febrile neutropenia[16], and comparable outcomes were also reported in a German study[17] and in Northern American non-white populations[18].

The primary strengths of this study include the truly real-world setup, covering all eligible patients in a reasonably large geographical region. Because there are no nongovernmental health care providers that offer cancer chemotherapy in the Southeast Region in Sweden, every patient who was given the therapy and met the inclusion criteria was included. Another additional value is that Swedish health care is generally available and publicly funded, meaning that all individuals, regardless of socioeconomic status, are offered similar treatment and follow-up programs. In the current era of novel therapeutic options in the early and late stages of PC, including targeted treatments such as radium 223[19] and lutetium-177 [^{177}Lu]-PSMA-617[20], it becomes increasingly important to assess the efficacy and tolerability of treatments already established in standard practice.

Table 5 Side-effects reported

	Total, *n* = 94 (%)
Reason for termination of treatment	
Completed as planned	74 (79)
Adverse event	11 (12)
Fatigue	5 (5)
Tumor progression	2 (2)
Patient preference	1 (1)
Other	1 (1)
Bone marrow toxicity	
Hemoglobin	
Any grade	16 (17)
≥ grade 3-4	0
White blood cell count	
Any grade	20 (21)
≥ grade 3-4	15 (16)
Neutrophil count	
Any grade	19 (20)
≥ grade 3-4	19 (20)
Grade missing	1
Platelet count	
Any grade	2 (2)
≥ grade 3-4	0
Unplanned hospitalization under and within 30 d after chemotherapy	24 (26)
Febrile neutropenia	20 (21)

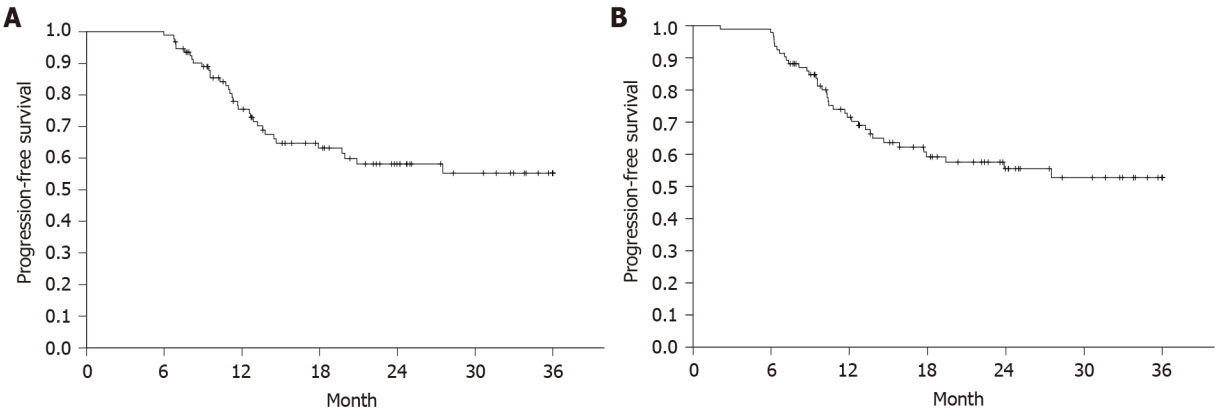


Figure 1 Progression-free survival according to the CHAARTED and STAMPEDE definitions of progressive disease and Swedish national guidelines. Time from date of diagnosis to last follow-up/death. Censored at 36 mo. A: CHAARTED and STAMPEDE; B: Swedish national guidelines.

The study's primary weakness mirrors its primary strength: The retrospective inclusion and, to some extent, different treatment regimens prescribed make it more difficult to define the efficacy and toxicity of the treatment schedule evaluated in the STAMPEDE and CHAARTED trials. The limited sample size means that subgroup analyses should be considered exploratory, and that their results should be interpreted with care.

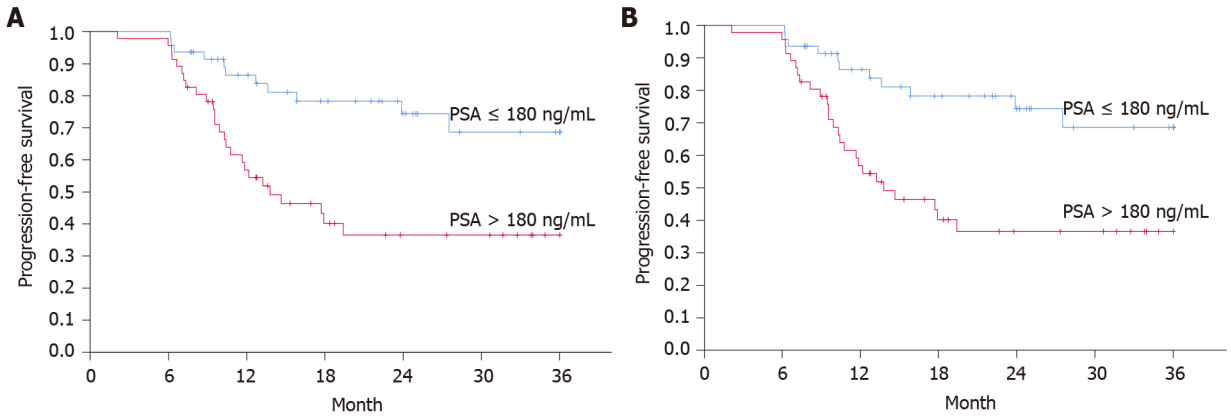


Figure 2 Progression-free survival for subgroups defined by PSA over/under median. A: Progression-free survival (PFS) according to the CHAARTED and STAMPEDE definitions of progressive disease (log-rank: $P = 0.0027$); B: PFS according to the Swedish national guidelines (log-rank: $P = 0.0018$). Time from date of diagnosis to last follow-up/death. Censored at 36 mo.

CONCLUSION

This study provides additional evidence on the efficacy and safety of upfront docetaxel in a real-world context of mCSPC. Progression-free and OS appear similar in real-world and randomized controlled trial settings. Febrile neutropenia remains a frequent and severe adverse event, and unplanned hospitalizations are common in patients undergoing this treatment. High baseline PSA indicates worse prognosis. In conclusion, results support the implementation of upfront docetaxel plus ADT as part of the SOC treatment strategy in mCSPC.

ARTICLE HIGHLIGHTS

Research background

Randomized phase-III trials indicate that upfront treatment with docetaxel, in addition to androgen deprivation therapy (ADT), improves survival in metastatic castration-sensitive prostate cancer (mCSPC). Less is known about the outcome of such treatment in real-world patients treated outside the frames of a clinical trial.

Research motivation

It is important to assess the outcome and safety of upfront docetaxel and ADT combination therapy following its implementation in real-world patients with mCSPC.

Research objectives

To evaluate the outcome of docetaxel and ADT combination therapy in real-world patients with mCSPC in terms of progression-free survival (PFS), overall survival (OS), and safety.

Research methods

A multicenter retrospective noninterventional study was performed and included 94 first consecutive real-world patients with mCSPC receiving upfront docetaxel and ADT in the Southeast Health Care Region of Sweden. Univariate and multivariate regression analyses were performed to identify prognostic parameters. Adverse events and unplanned hospitalizations were thoroughly reviewed.

Research results

PFS at 12 and 24 mo was 75% and 58%, while OS was 93% and 86% concurrently points, respectively. High baseline PSA levels were associated with worse prognosis in multivariate regression analysis. Twenty-one percent of the patients experienced febrile neutropenia, and 26% had at least one episode of unplanned hospitalization.

Research conclusions

The outcome and safety of docetaxel and ADT combination therapy in mCSPC appear

similar in real-world and randomized controlled trial populations. This study supports further implementation of this treatment strategy in standard of care.

Research perspectives

Future studies must identify clinically useful biomarkers and tools for tailored treatment strategies in patients with mCSPC.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Uptake and outcomes of small intestinal and urinary tract cancer surveillance in Lynch syndrome

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Abstract

BACKGROUND

Lynch syndrome (LS) is a hereditary cancer predisposition syndrome associated with increased risk of multiple cancers. While colorectal cancer surveillance decreases mortality in LS and is recommended by guidelines, there is lack of evidence for the efficacy of surveillance for extra-colonic cancers associated with LS, including small intestinal cancer (SIC) and urinary tract cancer (UTC). Given the limited evidence, guidelines do not consistently recommend surveillance for SIC and UTC, and it remains unclear how often individuals will choose to undergo and follow through with extra-colonic surveillance recommendations.

AIM

To study factors associated with SIC and UTC surveillance uptake and outcomes in LS.

METHODS

This is an IRB-approved retrospective analysis of individuals with LS seen at a tertiary care referral center. Included individuals had a pathogenic or likely pathogenic variant in *MLH1*, *MSH2*, *MSH6*, *PMS2*, or *EPCAM*, or were a confirmed obligate carrier, and had at least one documented visit to our center. Information regarding SIC and UTC surveillance was captured for each individual, and detailed personal and family history was obtained for individuals

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Grade B (Very good): 0
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Grade E (Poor): 0

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who had an initial LS management visit in our center's dedicated high-risk LS clinic between January 1, 2017 and October 29, 2020. During these initial management visits, all patients had in-depth discussions of SIC and UTC surveillance with 1 of 3 providers experienced in LS management to promote informed decision-making about whether to pursue SIC and/or UTC surveillance. Statistical analysis using Pearson's chi-squared test and Wilcoxon rank-sum test was completed to understand the factors associated with pursuit and completion of SIC and UTC surveillance, and a *P* value below 0.05 was deemed statistically significant.

RESULTS
Of 317 individuals with LS, 86 (27%) underwent a total of 105 SIC surveillance examinations, with 5 leading to additional work-up and no SICs diagnosed. Additionally, 99 (31%) patients underwent a total of 303 UTC surveillance examinations, with 19 requiring further evaluation and 1 UTC identified. Of 155 individuals who had an initial LS management visit between January 1, 2017 and October 29, 2020, 63 (41%) chose to undergo SIC surveillance and 58 (37%) chose to undergo UTC surveillance. However, only 26 (41%) and 32 (55%) of those who initially chose to undergo SIC or UTC surveillance, respectively, successfully completed their surveillance examinations. Individuals with a pathogenic variant in *MSH2* or *EPCAM* were more likely to initially choose to undergo SIC surveillance ($P = 0.034$), and older individuals were more likely to complete SIC surveillance ($P = 0.007$). Choosing to pursue UTC surveillance was more frequent among older individuals ($P = 0.018$), and females more frequently completed UTC surveillance ($P = 0.002$). Personal history of cancer and family history of SIC or UTC were not significantly associated with electing nor completing surveillance. Lastly, the provider discussing SIC/UTC surveillance was significantly associated with subsequent surveillance choices.

CONCLUSION
Pursuing and completing SIC/UTC surveillance in LS is influenced by several factors, however broad incorporation in LS management is likely unhelpful due to low yield and frequent false positive results.

Key Words: Lynch syndrome; Urinary tract cancer; Intestinal neoplasms; Early diagnosis of cancer; Patient preference; Gastrointestinal surgical procedure

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Core Tip: This retrospective study of a Lynch syndrome (LS) cohort measured the uptake and outcome of small intestinal cancer (SIC) and urinary tract cancer (UTC) surveillance. When given the option of surveillance, a minority of patients elected surveillance, and patient completion of surveillance exams was suboptimal. Completed surveillance exams rarely detected SIC/UTC and resulted in multiple false positives that led to additional follow-up procedures. Pursuing and completing SIC/UTC surveillance in LS was influenced by several factors, however given the low yield and positive predictive value, broad incorporation of SIC/UTC surveillance in LS management is unlikely to be helpful.

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INTRODUCTION

Lynch syndrome (LS) is an autosomal dominant cancer predisposition syndrome

resulting from a disease-causing variant in the *MLH1*, *MSH2*, *MSH6*, *PMS2*, or *EPCAM* gene. LS is primarily associated with increased colorectal and endometrial cancer risk but is also associated with increased risk of gastric, small intestinal, hepatobiliary, ovarian, urinary tract, brain, and skin cancers[1-3]. Colorectal cancer surveillance has proven effective in LS, with colonoscopy decreasing colorectal cancer mortality in LS by approximately 65%, which has led to inclusion of frequent colonoscopy in all LS management guidelines[4-8]. There is less agreement about the utility of surveillance for extra-colonic cancers in LS. Although recent reports show upper gastrointestinal cancer surveillance could detect gastric and duodenal cancers in LS[9,10], differences remain in recommended upper gastrointestinal cancer surveillance[5,11]. There is a paucity of evidence supporting efficacy of other types of extra-colonic cancer surveillance in LS, including small intestinal cancer (SIC) and urinary tract cancer (UTC) surveillance, as well as a lack of data addressing provider recommendation and patient uptake of SIC and UTC surveillance.

The cumulative risk of small intestinal adenocarcinoma in LS is up to 11%, markedly higher than the general population risk of 0.3%[5,7,12-14]. Options for SIC surveillance include small bowel follow through (SBFT), video capsule endoscopy (VCE), or CT/MRI enterography, with VCE being considered the most sensitive for small intestinal pathology[15,16]. However, there are limitations to the use of VCE, including cost and possible capsule retention in those with a history of prior abdominal surgery[17]. A study of SIC surveillance with VCE in 35 asymptomatic LS patients identified 2 adenomas and 1 adenocarcinoma, all distal to the duodenum[18]. A separate study of VCE performed on 200 asymptomatic LS patients led to the discovery of a small intestinal adenoma and a small intestinal adenocarcinoma[19]; this same group performed follow-up VCE on 155 patients of the same cohort a mean interval of 2 years after the first VCE which led to no additional small intestinal neoplasms being detected[20]. Other prospective and retrospective studies of LS cohorts concluded that the low frequency of SIC in LS prevented surveillance from being cost effective[20,21]. Given the limited data on SIC surveillance, there is currently no consensus recommendation regarding dedicated SIC surveillance for individuals with LS.

Individuals with LS are also at increased risk of UTC, including cancer of the renal pelvis, bladder, and ureters[21-23]. Cumulative risk of UTC in LS is up to 28%, a 20-fold increase in risk compared to the general population, with the highest risk seen in males with pathogenic *MSH2* variants[5,13,21,24,25]. Options for UTC surveillance include urinalysis, urine cytology, CT urogram, or cystoscopy. A study of the Danish HNPCC Registry found that only 2 (0.1%) of 1,868 urine cytology screens in 977 patients led to a diagnosis of an asymptomatic tumor, and 22 screens (1.2%) led to a false positive result, leading the authors to conclude that urine cytology is not an ideal surveillance method in LS[23]. Another study of the same registry found that 78% of UTCs in the cohort occurred in patients without a family history of UTC, and 73% of UTCs were in individuals with a pathogenic *MSH2* variant or a first degree relative of a *MSH2* carrier, leading the authors to suggest that UTC surveillance should not be limited to patients with a family history of UTC and should be focused on patients with pathogenic *MSH2* variants[25]. There has been disagreement amongst LS guidelines regarding the utility of UTC surveillance as some groups, like the U.S. Multi-Society Task Force, have recommended considering routine surveillance[6,26-29], while others, including the Mallorca group, have deemed there is not sufficient evidence to recommend regular surveillance[5,7,23,30].

Apart from whether SIC or UTC surveillance is recommended, it is equally important to understand whether individuals with LS will undergo surveillance if recommended, especially given the already intensive surveillance recommendations often mandated as part of a comprehensive LS surveillance program. Data characterizing extra-colonic cancer surveillance compliance in LS are limited, however a large study of annual UTC surveillance in LS found a compliance rate of only 29%[23]. This compliance is substantially lower than colonoscopy compliance (68%-85%)[31,32]. Furthermore, it remains uncertain what factors influence a patient's decision to pursue SIC or UTC surveillance in the presence of variable guidelines and recommendations.Herein, we aim to characterize the uptake and outcomes of SIC and UTC surveillance in LS, including patients' decisions about whether to pursue surveillance despite the limited evidence on efficacy and varying guideline recommendations, whether these individuals successfully complete surveillance, and the yield of the surveillance examinations.

MATERIALS AND METHODS

This is a retrospective study of individuals with LS seen at Penn Medicine, approved by the University of Pennsylvania Institutional Review Board. Individuals with LS had a confirmed pathogenic or likely pathogenic variant in *MLH1*, *MSH2*, *MSH6*, *PMS2*, or *EPCAM*, or were an obligate carrier of a familial pathogenic or likely pathogenic variant in one of these genes, and had at least one visit to the health system.

The electronic medical records of all LS patients were reviewed for pertinent details regarding demographics, medical history, family history, and surveillance examination results. Data were captured and entered into a REDCap database hosted at the University of Pennsylvania to facilitate statistical analysis. SIC surveillance was defined as a VCE or SBFT ordered in the absence of symptoms concerning for small intestinal pathology. Abnormal SIC surveillance results included any finding that was suspicious for a polyp or neoplastic process. UTC surveillance was defined as a urinalysis or urine cytology ordered in the absence of symptoms concerning for urinary tract pathology. Abnormal UTC surveillance results included a urine dipstick positive for blood, microscopic detection of red blood cells above the upper limit of normal, or atypical/abnormal urothelial cells found on urine cytology.

In an effort to further characterize recent SIC and UTC surveillance decisions, this study reviewed data from individuals who had their initial office visit for LS management in the Penn Medicine Gastrointestinal Cancer Genetics Program between January 1, 2017 and October 29, 2020 and were seen by 1 of 3 providers experienced in LS management. During the initial office visit to formulate the LS surveillance plan, each patient was engaged in detailed discussion about SIC and UTC surveillance covering the risks of surveillance (including false positive results generating additional evaluations), potential benefits, lack of robust data showing surveillance prevents cancer and/or reduces mortality, and lack of consistent guideline recommendations. If after this in-depth discussion a patient decides to pursue SIC and/or UTC surveillance, the provider orders appropriate testing and notes the patient's decision in their chart. If no evidence of a completed surveillance examination was in the patient's electronic medical record, the surveillance examination was noted as incomplete.

Statistical analysis using Pearson's chi-squared test for categorical variables and Wilcoxon rank-sum test for continuous variables was completed with a Type I error rate of 0.05 using Stata software version 16.1.

RESULTS

Three hundred seventeen individuals with LS had a visit to the health system and were included as part to the cohort; the cohort was mostly white (86%), non-Hispanic (98%), and female (59%), with a median age of 49 years (IQR: 38-61 years) (Supplementary Table 1). Distribution of LS genes in the cohort was relatively uniform as the percentage of pathogenic/likely pathogenic variants in *MLH1*, *MSH2*/*EPCAM*, *MSH6*, and *PMS2* was 23%, 35%, 22%, and 20% respectively. Of the 317 individuals with LS, 86 (27%) underwent a total of 105 SIC surveillance examinations with the majority (55%) being VCEs. There were no SICs diagnosed. However, 5 of these surveillance VCEs were suspicious for a small bowel polyp, which led to further work-up with invasive procedures (Table 1). None of the follow-up procedures led to the identification of any neoplastic small intestinal lesions. The positive predictive value (PPV) for VCE was 0% with a one-sided 97.5% confidence interval of 0%-52%. Of this same cohort of 317 individuals with LS, 99 (31%) underwent a total of 303 UTC surveillance examinations, the majority (65%) of which were urinalysis, with 19 of these surveillance tests showing abnormal findings that prompted further evaluation (Table 1). Of the 19 abnormal surveillance results leading to further work up, 10 (53%) were urine cytologies, and 1 surveillance urine cytology led to a single UTC diagnosis of a non-invasive high grade urothelial papillary carcinoma in a 64 year-old male individual with a pathogenic variant in *MSH2*. This patient had localized disease that was treated with nephroureterectomy and retroperitoneal/pelvic lymph node dissection and is subsequently followed by regular cystoscopy and MRI without reoccurrence. Urinalysis had a PPV of 0% with a one-sided 97.5% confidence interval of 0%-34%, and urine cytology had a PPV of 10% with a 95% confidence interval of 0.25%-45%.

To understand the factors influencing uptake of SIC and UTC surveillance in LS, we further analyzed those individuals with LS who had an initial LS management visit with our program between January 1, 2017 and October 29, 2020. This cohort was

Table 1 Small intestinal cancer and urinary tract cancer surveillance outcomes in Lynch syndrome

	***n* = 317**
Individuals who underwent SIC surveillance	86 (27%)
SIC surveillance exams completed per individual, median (IQR)	1 (1-1)
Total completed SIC surveillance exams	105
VCE	58 (55%)
SBFT	47 (45%)
Abnormal SIC surveillance exams leading to further work-up	5 (5%)
VCE	5 (100%)
SBFT	0 (0%)
Abnormal SIC surveillance exams leading to a SIC diagnosis	0 (0%)
Individuals who underwent UTC surveillance	99 (31%)
UTC surveillance exams completed per individual, median (IQR)	2 (1-3)
Total completed UTC surveillance exams	303
Urinalysis	197 (65%)
Urine cytology	106 (35%)
Abnormal UTC surveillance exams leading to further work-up	19 (6%)
Urinalysis	9 (47%)
Urine Cytology	10 (53%)
Abnormal UTC surveillance exams leading to a UTC diagnosis	1 (5%)

SIC: Small intestinal cancer; UTC: Urinary tract cancer; VCE: Video capsule endoscopy; SBFT: Small bowel follow through.

comprised of 155 individuals who were primarily white (90%) and female (65%), and the majority had private insurance (86%) (Table 2). There was a near equal distribution of patients across all LS genes, with 70 (45%) of these individuals having a personal history of cancer, including 2 (1%) with SIC and 6 (4%) with UTC. Almost all (97%) of these individuals had a family history of cancer, with 8 (5%) having a family history of SIC, and 35 (23%) having a family history of UTC. A majority of the cohort (78%) was treated by a single provider.

At their initial LS management visit, during which the risks and benefits of SIC and UTC surveillance were reviewed to allow patients to make an informed decision, 63 (41%) patients chose to undergo SIC surveillance and 58 (37%) chose to undergo UTC surveillance (Figure 1). However, of those who chose to undergo SIC and UTC surveillance at their initial management visit, only 26 (41%) and 32 (55%) completed their SIC or UTC surveillance examinations, respectively.

We next assessed for factors associated with choosing to undergo SIC and/or UTC surveillance as well as successfully completing surveillance tests. Individuals with a pathogenic variant in *MSH2* or *EPCAM* were more likely to initially choose to undergo SIC surveillance compared to those with other mutations as this group accounted for 24 (38%) of the individuals who chose SIC surveillance (Table 3, $P = 0.034$). Older age was associated with completion of SIC surveillance ($P = 0.007$), as the median age of those who completed SIC surveillance was 56 years—14 years higher than the median age of those who did not complete surveillance. Additionally, there were statistically significant differences in choosing and completing SIC surveillance in a provider-dependent manner.

For UTC surveillance, older age was associated with choosing to undergo surveillance; the median age of those who chose to undergo surveillance was 48 years, 8 years higher than those who chose no surveillance (Table 4, $P = 0.018$). Female sex was associated with UTC surveillance completion ($P = 0.002$) as 26 (81%) individuals who completed UTC surveillance were female. Ashkenazi Jewish ancestry was also associated with completion of UTC surveillance ($P = 0.006$). The individuals who were treated by Provider 1 chose UTC surveillance less frequently ($P = 0.000$) but were more likely to complete surveillance exams if they chose to pursue them ($P = 0.000$).

Table 2 Characteristics of patients with an initial Lynch syndrome management visit between January 1, 2017 and October 29, 2020

	***n* = 155**
Age (yr), median (IQR)	46 (33-58)
Female sex	100 (65%)
Race	
White	139 (90%)
Black	3 (2%)
Asian	7 (5%)
Other	2 (1%)
Unknown	4 (3%)
Hispanic ethnicity	2 (1%)
Ashkenazi Jewish ancestry	16 (10%)
Lynch syndrome gene	
MLH1	29 (19%)
MSH2 or *EPCAM*	40 (26%)
MSH6	45 (29%)
PMS2	41 (26%)
Personal history of cancer	70 (45%)
Small intestinal	2 (1%)
Urinary tract	6 (4%)
Colorectal	30 (19%)
Family history of cancer	151 (97%)
Small intestinal	8 (5%)
Urinary tract	35 (23%)
Colorectal	113 (73%)
Type of insurance	
Private insurance	134 (86%)
Medicare insurance	16 (10%)
Medicaid insurance	5 (3%)
Provider	
Provider 1	121 (78%)
Provider 2	17 (11%)
Provider 3	17 (11%)

Personal history of cancer and family history of SIC or UTC were not associated with initially choosing to undergo surveillance or surveillance completion (Tables 3-4). Race, Hispanic ethnicity, and insurance status were also not associated with choosing nor completing surveillance (Tables 3-4).

DISCUSSION

Lynch syndrome is a high-risk cancer predisposition syndrome, with affected individuals requiring lifelong cancer risk management. Whereas some surveillance, such as colorectal cancer surveillance, is strongly recommended in LS, there is a lack of consistent recommendations for SIC and UTC surveillance due to the limited data showing this extra-colonic surveillance is effective. In this study, we investigated uptake of SIC and UTC surveillance in LS and the outcomes of the associated

Table 3 Characteristics of individuals with Lynch syndrome who chose to undergo and/or completed small intestinal cancer surveillance

	Surveillance chosen (*n* = 63)	Surveillance not chosen (*n* = 92)	*P* value	Surveillance completed (*n* = 26)	Surveillance not completed (*n* = 37)	*P* value
Age (yr), median (IQR)	48 (37-59)	42 (32-57)	0.114	56 (46-62)	42 (33-54)	0.007[a]
Female	44 (70%)	56 (61%)	0.252	21 (81%)	23 (62%)	0.113
Race			0.750			0.531
White	58 (92%)	81 (88%)		24 (92%)	34 (92%)	
Black	1 (2%)	2 (2%)		0 (0%)	1 (3%)	
Asian	3 (5%)	4 (4%)		2 (8%)	1 (3%)	
Other	0 (0%)	2 (2%)		-	-	
Unknown	1 (2%)	3 (3%)		0 (0%)	1 (3%)	
Hispanic ethnicity	1 (2%)	1 (1%)	0.912	0 (0%)	1 (3%)	0.211
AJ ancestry	5 (8%)	11 (12%)	0.687	1 (4%)	4 (11%)	0.489
Lynch syndrome gene			0.034[a]			0.357
MLH1	10 (16%)	19 (21%)		5 (19%)	5 (14%)	
MSH2 or *EPCAM*	24 (38%)	16 (17%)		7 (27%)	17 (46%)	
MSH6	14 (22%)	31 (34%)		8 (31%)	6 (16%)	
PMS2	15 (24%)	26 (28%)		6 (23%)	9 (24%)	
Personal history of cancer	33 (52%)	37 (40%)	0.135	17 (65%)	16 (43%)	0.083
Family history of SIC	5 (8%)	3 (3%)	0.182	1 (4%)	4 (11%)	0.340
Insurance			0.111			0.314
Private	58 (92%)	76 (83%)		25 (96%)	33 (89%)	
Medicare	5 (8%)	11 (12%)		1 (4%)	4 (11%)	
Medicaid	0 (0%)	5 (5%)		-	-	
Provider			0.000[a]			0.030[a]
Provider 1	39 (62%)	82 (89%)		17 (65%)	22 (59%)	
Provider 2	13 (21%)	4 (4%)		8 (31%)	5 (14%)	
Provider 3	11 (17%)	6 (7%)		1 (4%)	10 (27%)	

[a]$P < 0.05$. SIC: Small intestinal cancer; AJ: Ashkenazi Jewish.

surveillance examinations. Our data shows that after engagement in an in-depth discussion on the potential benefits, risks, and limitations of SIC and UTC surveillance, a majority of patients decided to forgo surveillance, and those that initially chose to pursue surveillance had low completion rates. Additionally, we show a low PPV with frequent false positive results for both SIC and UTC surveillance, and the overall yield of cancer diagnoses was low for all surveillance methods. Taken together, our results do not support regular incorporation of SIC and UTC surveillance into standard LS cancer risk management care.

Effective cancer surveillance in LS should ideally utilize testing that is cost-effective and low risk, and surveillance should ultimately increase survival[7]. An ideal test must also have a high level of sensitivity and specificity, as false positive cancer surveillance results not only lead to further work-up but also lead to emotional distress for the patient as well as decreased compliance rates and follow-up with subsequent surveillance exams[33,34]. Additionally, false positive results can expose patients to possible harms resulting from superfluous follow-up procedures as well as

Table 4 Characteristics of individuals with Lynch syndrome who chose to undergo and/or completed urinary tract cancer surveillance

	Surveillance chosen (*n* = 58)	Surveillance not chosen(*n* = 97)	*P* value	Surveillance completed (*n* = 32)	Surveillance not completed (*n* = 26)	*P* value
Age (yr), median (IQR)	48 (39-60)	40 (32-55)	0.018[a]	45 (40-61)	50 (37-60)	0.772
Female	37 (64%)	63 (65%)	0.884	26 (81%)	11 (42%)	0.002[a]
Race			0.431			0.498
White	54 (93%)	85 (88%)		30 (94%)	24 (92%)	
Black	1 (2%)	2 (2%)		0 (0%)	1 (4%)	
Asian	3 (5%)	4 (4%)		2 (6%)	1 (4%)	
Other	0 (0%)	2 (2%)		-	-	
Unknown	0 (0%)	4 (4%)		-	-	
Hispanic ethnicity	0 (0%)	2 (2%)	0.336	-	-	-
AJ ancestry	3 (5%)	13 (13%)	0.229	3 (9%)	0 (0%)	0.006[a]
Lynch syndrome gene			0.640			0.171
MLH1	8 (14%)	21 (22%)		3 (9%)	5 (19%)	
MSH2 or *EPCAM*	17 (29%)	23 (24%)		9 (28%)	8 (31%)	
MSH6	17 (29%)	28 (29%)		13 (41%)	4 (15%)	
PMS2	16 (28%)	25 (26%)		7 (22%)	9 (35%)	
Personal history of cancer	29 (50%)	41 (42%)	0.349	17 (53%)	12 (46 %)	0.597
Family history of UTC	15 (26%)	20 (21%)	0.477	11 (34%)	4 (15%)	0.118
Insurance			0.631			0.402
Private	50 (86%)	84 (87%)		29 (91%)	21 (81%)	
Medicare	7 (12%)	9 (9%)		3 (9%)	4 (15%)	
Medicaid	1 (2%)	4 (4%)		0 (0%)	1 (4%)	
Provider						
Provider 1	36 (62%)	85 (88%)	0.001[a]	28 (88%)	8 (31%)	0.000[a]
Provider 2	12 (21%)	5 (5%)		2 (6%)	10 (38%)	
Provider 3	10 (17%)	7 (7%)		2 (6%)	8 (31%)	

[a]$P < 0.05$. UTC: Urinary tract cancer; AJ: Ashkenazi Jewish.

additional medical costs related to these procedures. Our data showed that both SIC and UTC surveillance had a low PPV, with 24 positive surveillance studies leading to the diagnosis of only 1 neoplastic lesion. These data are consistent with a previous study of SIC screening in asymptomatic LS patients that observed 13 VCE results suspicious for SIC, none of which led to the confirmation of a SIC through follow-up testing[20]. While VCE is the most sensitive test for picking up small intestinal pathology[15,16], it may have a downside of being less specific, leading to high rates of false positive test results that require patients to undergo unnecessary invasive procedures. In addition to this observed low specificity, the sensitivity of urine cytology in asymptomatic LS patients has also been previously reported to be poor (29%)[23]. Effective cancer surveillance should also result in improved survival. With only one individual in our study having a surveillance-detected UTC or SIC, we are unable to meaningfully comment on the impact of surveillance on cancer survival, however at this time it remains unclear if early diagnosis of UTC or SIC leads to higher survival rates[7,35]. Together, the low yield and low PPV of SIC and UTC surveillance described in this study do not provide support for broad inclusion of SIC and/or UTC surveillance in LS management; however, whether this surveillance should be

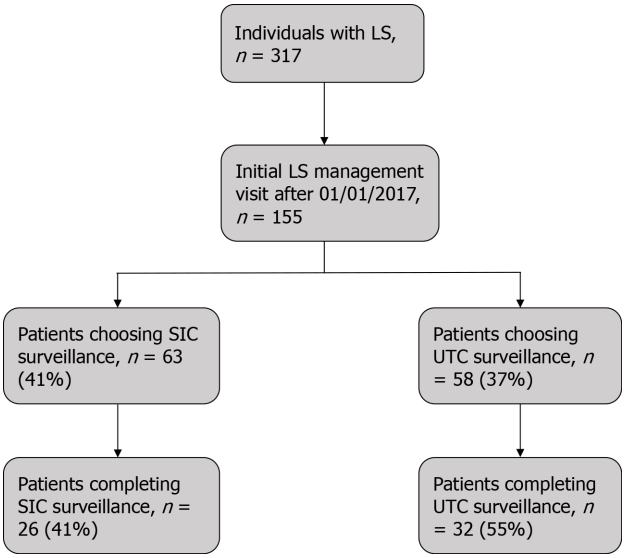


Figure 1 Schematic representation of small intestinal cancer and urinary tract cancer surveillance uptake among individuals with LS. SIC: Small intestinal cancer; UTC: Urinary tract cancer; LS: Lynch syndrome.

considered for certain sub-groups of patients with LS will require future larger studies.

The majority of the patients in our cohort chose not to pursue SIC or UTC surveillance after having an in-depth discussion with their provider about the risks, benefits, and limitations of surveillance. It is likely that this discussion and the provider involved influenced the patients' decision making; accurate risk perception has previously been observed to impact LS patients' behaviors towards cancer surveillance[36]. The difference in cancer surveillance behaviors between patients of different providers could result from differing manners in which the providers discussed surveillance options in an individual's initial LS management visit. In this study, individuals with pathogenic variants in *MSH2* or *EPCAM* chose to pursue SIC surveillance more frequently, while older individuals chose to pursue UTC surveillance more often. In a study of colorectal cancer survivors with Lynch-like syndrome, increased cancer worry was associated with a stronger belief that extra-colonic cancer surveillance was necessary[37]; perhaps learning of the increased cancer risk that comes with age and pathogenic *MSH2* or *EPCAM* variants compelled patients in this study to opt for additional surveillance. The observation that individuals with *MSH2* or *EPCAM* pathogenic variants were more likely to choose to undergo SIC surveillance, but not UTC surveillance, compared to individuals with pathogenic variants in other genes may be due to a Type I error or may also be influenced by other factors that were not captured in this study. The choice to initially pursue surveillance was likely also influenced by other factors not examined in this study. Some individuals may have decided to forgo surveillance due to the emotional distress that comes with the increased surveillance burden and the requirement to navigate potentially challenging health care system infrastructures, factors that have been shown to influence other cancer surveillance in LS[36]. Other variables such as associated costs and familial obligations may also have affected patient decision making. A future prospective study surveying the attitudes and perceptions of individuals with LS about low-evidence surveillance tests would be important to help answer this question.

The completion rate of SIC and UTC surveillance in this cohort was 41% and 55%, respectively, which is higher than a previous study of compliance with UTC surveillance in LS finding a rate of 29%[23]. Our increased completion rate may have resulted from the in-depth discussion on this surveillance between the patient and provider. However, our observed completion rate was lower than the reported compliance rate for colonoscopy within the LS population (68-85%)[31,32]. Colorectal cancer risk is well-recognized as one of the highest cancer risks in LS, and therefore, the lower completion rate compared to colorectal cancer surveillance may be due to the individuals' perception of the decreased risk of extra-colonic cancers[36]. The discrepancy could also be due to provider emphasis on the effectiveness of colonoscopy to decrease mortality and morbidity. In this study, we observed that providers may influence an individual's choices towards SIC and UTC surveillance, both in terms of choosing to undergo surveillance and completing surveillance.

Additionally, we found completion of SIC surveillance was more frequent among older individuals, and completion of UTC surveillance was more frequent among those of female sex and Ashkenazi Jewish ancestry. These findings present a contrast to another study of a LS cohort which observed that cancer surveillance completion was associated with younger age[38]. This other study also ascertained that surveillance completion was influenced by occupation status, a factor not captured by this study. Other unexamined factors could have played a role in surveillance completion, as well; for instance, the completion of surveillance may have been put on hold due to the management of other ongoing health issues.

Considering the limited information available on the effectiveness of SIC and UTC surveillance in LS, which was further obfuscated by the findings of this study, we do not believe that SIC and UTC surveillance should be broadly performed in all individuals with LS. Instead, we advocate for the individualized incorporation of these surveillance methods in a patient-dependent manner after a detailed discussion of the risks, limitations, benefits, and uncertainties. A larger prospective study would be better equipped to assess the true benefits and risks of SIC and UTC surveillance as well as to understand patients' interest in and concerns with extra-colonic surveillance. Additionally, the low PPV of the surveillance methods observed in this study emphasize the need for further research on the cost of this surveillance and the effect of early detection of SIC and UTC on patient morbidity and mortality. Qualitative studies could also elucidate patient perspectives as individuals with LS may have negative psychological effects if multiple extra-colonic cancer surveillance studies are incorporated into their management.

Limitations of this study include that the LS cohort is from a single tertiary care center and lacks racial diversity; therefore, the results observed may not be representative of more geographically and racially diverse cohorts. Another limitation is that individuals may have completed SIC or UTC surveillance outside of our medical center, with these completed surveillance tests neither appearing in the individual's electronic medical record nor being captured by this study. Finally, this study has a relatively small sample size, which may prevent recognition of other significant associations.

CONCLUSION

This cohort study describes outcomes of SIC and UTC surveillance in LS and identifies factors influencing the SIC and UTC surveillance practices of individuals with LS. This study highlights problems with incorporation of SIC and UTC surveillance into LS care, as illustrated by the low PPV and low overall yields of these tests. The study also shows that the pursuit and completion of these surveillance examinations may depend on the affected individual's age, sex, genotype, and provider; however at this time, there is insufficient evidence to support widespread use of SIC/UTC surveillance in all individuals with LS. Further large-scale studies on SIC and UTC surveillance are needed to better understand the utility of available surveillance tests as well as their cost effectiveness and impact on patient survival.

ARTICLE HIGHLIGHTS

Research background

Lynch syndrome (LS) is an autosomal dominant cancer predisposition syndrome resulting from a disease-causing variant in the *MLH1*, *MSH2*, *MSH6*, *PMS2*, or *EPCAM* gene. LS is primarily associated with increased colorectal and endometrial cancer risk, but it is also associated with increased risk of small intestinal cancer (SIC) and urinary tract cancer (UTC). Cancer surveillance management for SIC and UTC has yet to be standardized for LS patients due to a lack of proven efficacy for current surveillance methods, and data regarding provider and patient interest in the current SIC and UTC surveillance methods are also lacking.

Research motivation

This study was interested in describing the efficacy and impact of completed SIC and UTC surveillance exams in a cohort of 317 LS patients. In addition, we were interested in patients' decisions about whether to pursue surveillance despite the limited evidence on efficacy and varying guideline recommendations and whether these

individuals successfully completed surveillance.

Research objectives

To characterize the uptake and outcomes of SIC and UTC surveillance among LS patients at a tertiary care referral center. We intended to analyze the factors influencing individuals' surveillance behaviors and to calculate the yield of completed surveillance exams.

Research methods

This was a retrospective study of individuals with LS seen at a tertiary care referral center. Information regarding SIC and UTC surveillance was captured for each individual. Additional demographic information and medical history was collected for individuals who had an initial LS management visit in our center's dedicated high-risk LS clinic between January 1, 2017 and October 29, 2020 to allow for analysis of individuals' behaviors after engaging in an in-depth conversation regarding surveillance with a provider in the clinic. Statistical analysis using Pearson's chi-squared test and Wilcoxon rank-sum test was completed, and a *P* value below 0.05 was deemed statistically significant.

Research results

Of the 317 individuals with LS in our cohort, 27% underwent a total of 105 SIC surveillance exams, and 31% underwent a total of 303 UTC surveillance exams. Each surveillance method was found to have a low positive predictive value and yield. A single UTC was diagnosed, and 0 SICs were diagnosed. Of 155 individuals who had an initial LS management visit between January 1, 2017 and October 29, 2020, a minority of individuals chose to undergo either SIC (41%) or UTC (37%) surveillance. Only 41% of individuals completed SIC surveillance, and 55% completed UTC surveillance when ordered. Several factors were found to be significantly associated with surveillance pursuit and completion, including age, sex, genotype, and provider.

Research conclusions

This study observed a low positive predictive value and yield for completed SIC and UTC surveillance exams, and after an in-depth conversation on the limitations and benefits of SIC and UTC surveillance, there was limited interest for this surveillance among individuals with LS. At this time, there continues to be insufficient evidence to support widespread SIC and UTC surveillance in LS.

Research perspectives

This study highlights the need for further research in SIC and UTC surveillance in LS. More data is needed on the cost of SIC and UTC surveillance and the effect of early detection of SIC and UTC on patient morbidity and mortality. Qualitative studies are also needed to elucidate patient perspectives regarding the addition of low-evidence surveillance exams to their cancer surveillance management.

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ORIGINAL ARTICLE

Retrospective Study

First-line pazopanib in patients with advanced non-clear cell renal carcinoma: An Italian case series

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Abstract

BACKGROUND

Non-clear cell (ncc) metastatic renal-cell carcinoma (RCC) has dismal results with standard systemic therapies and a generally worse prognosis when compared to its clear-cell counterpart. New systemic combination therapies have emerged for metastatic RCC (mRCC), but the pivotal phase III trials excluded patients with nccRCC, which constitute about 30% of metastatic RCC cases.

AIM

To provide a piece of real-life evidence on the use of pazopanib in this patient subgroup.

METHODS

The present study is a multicenter retrospective observational analysis aiming to assess the activity, efficacy, and safety of pazopanib as first-line therapy for advanced nccRCC patients treated in a real-life setting.

RESULTS

Overall, 48 patients were included. At the median follow-up of 40.6 mo, the objective response rate was 27.1%, the disease control rate was 83.3%, and the median progression-free survival and overall survival were 12.3 (95% confidence interval [CI]: 3.6-20.9) and 27.7 (95%CI: 18.2-37.1) mo, respectively. Grade 3 adverse events occurred in 20% of patients, and no grade 4 or 5 toxicities were found.

CONCLUSION

Pazopanib should be considered as a good first-line option for metastatic RCC with variant histology.

Key Words: Pazopanib; Non-clear cell; Kidney cancer; Renal-cell carcinoma; Variant histology; Tyrosine kinase inhibitors

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Core Tip: Non-clear cell metastatic renal-cell carcinoma (nccRCC) has dismal results with standard systemic therapies and a poor prognosis. Few therapeutic molecules have

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been explicitly tested in nccRCC patients. We retrospectively collected 48 advanced nccRCC patients treated with pazopanib in the first-line setting, offering promising findings of quite good response rate (27%), progression-free survival around 12 mo, and overall survival around 28 mo. In light of these results, we suggest that pazopanib can be a good treatment choice in this subgroup of patients, pending the results of ongoing clinical trials with new therapeutic combinations.

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INTRODUCTION

Non-clear cell renal-cell carcinoma (nccRCC) represents a heterogeneous group of tumors with distinct genomic and metabolic features. Therefore, its clinical behavior can be benign to indolent and even highly malignant with high metastatic potential[1].

Of note, non-clear mRCC has dismal results with standard systemic therapies and a generally worse prognosis when compared to its clear-cell counterpart, as demonstrated in a large study by the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC), showing a worse overall survival (OS) for patients with nccRCC compared to ccRCC patients [12.8 mo (95%CI: 11.0-16.1) *vs* 22.3 mo (20.7-23.5)][2].

Recent advances have offered the availability of new systemic therapies for metastatic RCC (mRCC), such as immunotherapy-based combinations with the current recommendation in the first-line setting[3-6]. These combinations, all forecasting the use of anti-PD-1/PD-L1 immune checkpoint inhibitors (ICIs), have been investigated in phase III randomized clinical trials (RCTs) enrolling patients with clear-cell RCC (ccRCC) or at least a clear-cell component in the tumor histology. Nevertheless, conventional clear-cell RCC accounts for only 70% of renal cortical tumors that metastasize, preventing to provide evidence for 30% of mRCC patients still lacking indication for the most productive therapeutic solutions. About nccRCC, international guidelines still recommend that such patients should be preferentially referred to clinical trials.

The previous gold-standard first-line therapies for mRCC, represented by anti-vascular endothelial growth factor receptor tyrosine kinase inhibitors (TKIs) used as a single agent, have been in part investigated in nccRCC patients. In particular, sunitinib was tested in two prospective trials (the ESPN trial and the ASPEN trial) initially planned to show the superiority of the mammalian target of rapamycin inhibitor everolimus over sunitinib as first-line therapy. Both studies, on the contrary, finally supported the use of sunitinib as a primary systemic approach in this population of patients[7,8]. On the other hand, other TKIs have been approved in the first-line setting for all-mRCC histologies, including the alternative with pazopanib, which demonstrated similar efficacy and even better safety profile when compared to sunitinib in randomized trials[9,10]. Nevertheless, little evidence is available about using this drug in nccRCC patients, with a consequent reluctance to prescription in clinical practice, notwithstanding the drug's good profile, also suitable for frail and elderly patients[11,12].

Given the unmet oncological need for evidence for the systemic approach to nccRCC, the present report of a retrospective multicenter case series aims to provide a piece of real-life evidence on the use of pazopanib in this patient subgroup.

MATERIALS AND METHODS

Study population and setting

The present study is a retrospective, observational analysis aiming to assess the

activity, efficacy, and safety of pazopanib as first-line therapy for advanced nccRCC patients treated in a real-life setting at multiple Italian institutions. The principal inclusion criteria were the diagnosis of nccRCC, including papillary RCC (pRCC), chromophobe RCC (chRCC), RCC with Xp11 translocation, RCC with undefined histology, and mixed-histology RCC with mostly ncc component; receiving the first dose of pazopanib between June 2012 and June 2015; > 18 years old; measurable disease at the computed tomography (CT) scans performed according to clinical practice at the treating centers. Data were collected between February 2017 and February 2018.

Study endpoints

The primary objective was to assess the outcome of patients in terms of objective response rate (ORR), disease control rate (DCR), progression-free survival (PFS), OS, and tolerability as co-primary endpoints. PFS was defined as the time between pazopanib initiation and disease progression or death; OS was defined as the time between pazopanib initiation and death or the date of the last follow-up visit for alive patients; DCR as responses plus stable diseases. Objective responses (complete, CR; partial, PR; stable, SD; progressive disease, PD) were evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST 1.1)[13] and assessed every 2-3 mo according to clinical practice.

Treatment-related adverse events (TRAEs) were recorded as clinical practice according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0 (in use at the time of the study conduct)[14].

The characteristics of patients were collected, and their correlation with the outcome was explored.

The study was reviewed and approved by the Local Ethics Committee of Parma (Italy). All study participants provided informed written consent before study enrollment.

Statistical analysis

Descriptive statistics are used to summarize the data. PFS and OS were estimated using the Kaplan-Meier method with 95% confidence intervals (CI) and compared using the log-rank test. Univariate and multivariate analyses were performed by using Cox proportional hazards models. The comparison between categorical endpoints was performed using the chi-square test. Significance levels were set at a 0.05 value, and all *P* values were two-sided. SPSS Statistics 24.0 software (IBM Corporation, Armonk, NY, United States) was used to conduct the statistical analyses.

RESULTS

From January 2011 to January 2017, 48 consecutive patients were included in 20 Italian centers. The median follow-up was 40.6 mo (95%CI: 22.3-58.9). The characteristics of patients are reported in Table 1. The median age was 70 (range, 27-86) years, and most patients were male (75.0%). Fifteen patients (31.3%) had distant metastases at disease onset. The majority of patients had pRCC (50.0%) or chRCC (18.8%) as histology. Most patients had previously received nephrectomy (85.4%), and seven (14.6%) had metastasectomy. Thirty-seven patients (77.1%) had an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0. All IMDC risk groups were represented in the study population.

The median duration of treatment was 9.1 (range, 0.6-52.5) mo, and eight patients (16.7%) were still receiving pazopanib at the time of the last follow-up. Twenty-eight patients (58.3%) received a second-line therapy (four received nivolumab and three cabozantinib).

In 17 cases (35.4%), the starting dose of pazopanib was primarily reduced because of the patient conditions (age and/or comorbidity): Ten patients started with 600 mg, and seven patients started with 400 mg. Secondary dose reductions or temporary treatment discontinuations due to TRAEs were required in 19 cases (39.6%), mainly due to grade (G) 3 hepatic toxicity, fatigue, and diarrhea. G3 TRAEs occurred in 20.8% of patients, G1-2 in 81.2%, and no G4 toxicity was observed (Table 2).

PR was achieved in 13 patients (27.1%), and no CR was observed. Twenty-seven patients (56.3%) obtained SD; the DCR was 83.3%, whereas six patients (12.5%) had PD as the best response (while 4.2% were not evaluable). Neither ORR nor DCR was significantly correlated with any of the following parameters: Sex, histology, grading, sarcomatoid component, initial pazopanib dose, ECOG PS, and IMDC risk group.

Table 1 Characteristics of patients, *n* (%)

Baseline characteristic	*n* = 48
Age, median (range)	70 (27-86)
Sex	
Males	36 (75)
Females	12 (25)
Histology	
Papillary	24 (50)
Chromophobe	9 (10.8)
Xp11 translocation	1 (2.1)
Unclassified	6 (12.5)
Mixed[1]	8 (16.7)
Grade (Fuhrman/ISUP)	
1-2	6 (12.5)
3	15 (31.3)
4	4 (8.3)
NA	23 (47.9)
Stage at diagnosis	
I-III	33 (68.8)
IV	15 (31.3)
Previous nephrectomy	
Yes	41 (85.4)
No	7 (14.6)
Metastasectomy	
Yes	7 (14.6)
No	41 (85.4)
ECOG PS	
0	37 (77.1)
1	11 (22.9)
IMDC score risk group	
Good	19 (39.6)
Intermediate	25 (52.1)
Poor	2 (4.2)
NA	2 (4.2)
Starting dose of pazopanib	
800 mg	31(64.6)
600 mg	10 (20.8)
400 mg	7 (14.6)

[1]Four tumors had mixed histology (clear-cell/papillary) with papillary histology prevalence and four tumors had mixed histology (clear-cell/chromophobe) with chromophobe histology prevalence.

NA: Not assessed/available; IMDC: International Metastatic Renal Cell Carcinoma Database Consortium; ECOG PS: Eastern Cooperative Oncology Group Performance Status; ISUP: International Society of Urological Pathology.

Table 2 Adverse events according to Common Terminology Criteria for Adverse Events (version 4.0)

Adverse event	Grade 1/2, *n* (%)	Grade 3, *n* (%)
Fatigue	24 (50.0)	
Diarrhea	15 (31.3)	2 (4.2)
Mucositis	9 (18.8)	
Hypertransaminasemia	7 (14.6)	4 (8.3)
Thrombocytopenia	6 (12.5)	
Anemia	15 (31.2)	2 (4.2)
Neutropenia	5 (10.4)	
Hypothyroidism	15 (31.3)	
Disgeusia	5 (10.4)	1 (2.1)
Cutaneous toxicity[1]	8 (16.7)	
Nausea/vomiting	14 (29.2)	
Heart failure	1 (2.1)	
Renal failure	5 (10.4)	1 (2.1)
Other[2]	16 (33.3)	

[1]Including discoloration of hairs and cutis, hand-foot syndrome, and dermatitis.
[2]Including bleeding, sleepiness, hypertrigliceridemia, hypophosphoremia, hyperbilirubinemia, loss of appetite/hyporexia, dyspepsia/epigastralgia, and hypertension.

Most cases of primary refractory (PD as best response) were pRCC. Table 3 shows the responses according to the histology.

Median PFS and OS were 12.3 (95%CI: 3.6-20.9) and 27.7 (95%CI: 18.2-37.1) mo, respectively (Figure 1, Table 3).

In the univariate analysis, no factors (among sex, previous nephrectomy, histology, grading, metastatic disease at diagnosis, previous metastasectomy, IMDC risk group, ECOG PS, and initial pazopanib dose) were significantly associated with PFS. Conversely, factors significantly associated with a better OS were IMDC group (P = 0.011), previous nephrectomy (P = 0.002), previous metastasectomy (P = 0.008), absence of metastatic disease at diagnosis (P = 0.014), and subsequent therapy with cabozantinib or nivolumab (P = 0.049).

Notwithstanding the limit of multivariate analysis in such a small sample size, it was performed for OS, and only the absence of metastatic disease at diagnosis (hazard ratio = 8.49, 95%CI: 1.76-40.90; P = 0.008) maintained a positive impact on OS.

DISCUSSION

The present analysis reported the most extensive case series, to our knowledge, treated with the TKI pazopanib as first-line systemic therapy for advanced nccRCC patients.

The activity and efficacy outcomes are in line with those reported by two prior series from the literature, with an ORR around 27%, a good DCR (over 80%), an mPFS over 1 year, and a good mOS of 27.7 mo. Compared to the other older reports, our study also included patients receiving new-generation drugs as second-line after pazopanib (*i.e.*, nivolumab or cabozantinib), suggesting (in the univariate analysis) this choice as significantly associated with improved survival and providing some evidence for these treatment sequences in nccRCC.

Regarding safety, pazopanib was generally well-tolerated, with G3 TRAEs occurring only in 20% of patients and no G4-5 toxicities. The AE-related discontinuations and the initial dose reductions were relatively frequent. Still, no impact on the outcome was evidenced for the latter choice, often preferable for frail patients with hepatic impairment or cardiovascular comorbidities.

The limitations of our report are represented by the retrospective nature, the heterogeneous follow-up time (with a wide range, but a good median value), the lack of central independent revision for the histological samples (nccRCC histology based on

Table 3 Objective response rate, progression-free survival, and overall survival outcomes

Effectiveness outcome	All patients (*n* = 48)	Papillary RCC (*n* = 24)	Chromophobe RCC (*n* = 9)
Response rate (RECIST 1.1), *n* (%)			
Partial responses	13 (27.1)	6 (25.0)	2 (22.2)
Complete responses	0 (0.0)	0 (0.0)	0 (0.0)
Stable disease	27 (56.3)	14 (58.3)	5 (55.5)
Progressions of disease	6 (12.5)	3 (12.5)	1 (11.1)
Not evaluable	2 (4.2)	1 (4.2)	1 (11.1)
Disease control rate	40 (83.3)	20 (83.3)	7 (77.8)
PFS			
Median (mo) (95%CI)	12.3 (3.6-20.9)	-	-
Rate of patients progression free at 6 mo	67.8%	-	-
Rate of patients progression free at 12 mo	49.0%	-	-
OS			
Median (mo) (95%CI)	27.6 (18.3-37.1)	-	-
Rate of patients alive at 12 mo	82.7%	-	-
Rate of patients alive at 24 mo	62.0%	-	-

RCC: Renal cell carcinoma; PFS: Progression free survival; OS: Overall survival; RECIST 1.1: Response Evaluation Criteria in Solid Tumours version 1.1.

the original diagnosis), and the limited number of patients receiving new-generation drugs (such as cabozantinib or nivolumab) in other treatment lines. On the other hand, the present analysis corroborates with the largest sample size two previous similar retrospective case series, providing herein clean data for pure first-line setting (as a difference *vs* Matrana *et al*[12]), and supporting the use of pazopanib as a single agent for nccRCC patients with advanced disease.

A further single-TKI alternative could be available in the next future as a primary choice for the subgroup of pRCC patients, represented by the third generation TKI cabozantinib. Currently approved as a first-line treatment option for ccRCC patients with poor- or intermediate-risk according to the IMDC model, this drug was investigated in a prospective study as a first- or second-line TKI for advanced pRCC. It demonstrated improved PFS (median 9.0 mo, 95%CI: 6-12) *vs* the comparator sunitinib (5.6 mo, 95%CI: 3-7; HR = 0.60, 95%CI: 0.37-0.97, $P = 0.019$) and ORR (23% *vs* 4% for sunitinib, $P = 0.010$), at the cost of quite manageable toxicity and a toxic death reported [15].

Beyond TKIs, the advent of immunotherapy has finally landed also in the field of nccRCC, reporting initial findings from prospective monotherapy trials. The single-arm phase II Keynote-427 trial investigated the efficacy and safety of single-agent pembrolizumab, a PD-1 inhibitor, in advanced nccRCC. In this study, 71.5% of patients had confirmed pRCC, 12.7% had chRCC, and 15.8% had unclassified RCC histology. Overall, the ORR was 26.7% (28.8% for papillary, 9.5% for chromophobe, and 30.8% for unclassified RCC); the mPFS was 4.2 mo (95%CI: 2.9-5.6), and the mOS was 28.9 mo. The toxicity was manageable, with 69.7% of patients reporting TRAEs; nevertheless, two deaths were reported as treatment-related[16]. These findings are pretty encouraging about the activity of immunotherapy in variant histologies. Anyway, the activity seems in line with those reported herein for pazopanib (similar ORR). In contrast, the efficacy appears relatively poor in terms of mPFS compared with that reported with TKIs in ours and similar populations. On the other hand, the OS outcome was similar.

Another first-line trial (Checkmate-920) recently investigated an immunotherapy combining ipilimumab and nivolumab (anti-CTLA-4 and anti-PD1 respectively) in a nccRCC treatment-naïve population. The first results reported relatively high rates of G3 (92.3%) and G4 (36.5%) toxicities (but without toxic deaths), ORR of 19.6%, and mPFS of 3.7 mo (95%CI: 2.7-4.6)[17]. The SUNNIFORECAST trial, a phase II randomized study currently ongoing with the same combination *vs* standard of care for previously untreated nccRCC patients, will provide further evidence about this

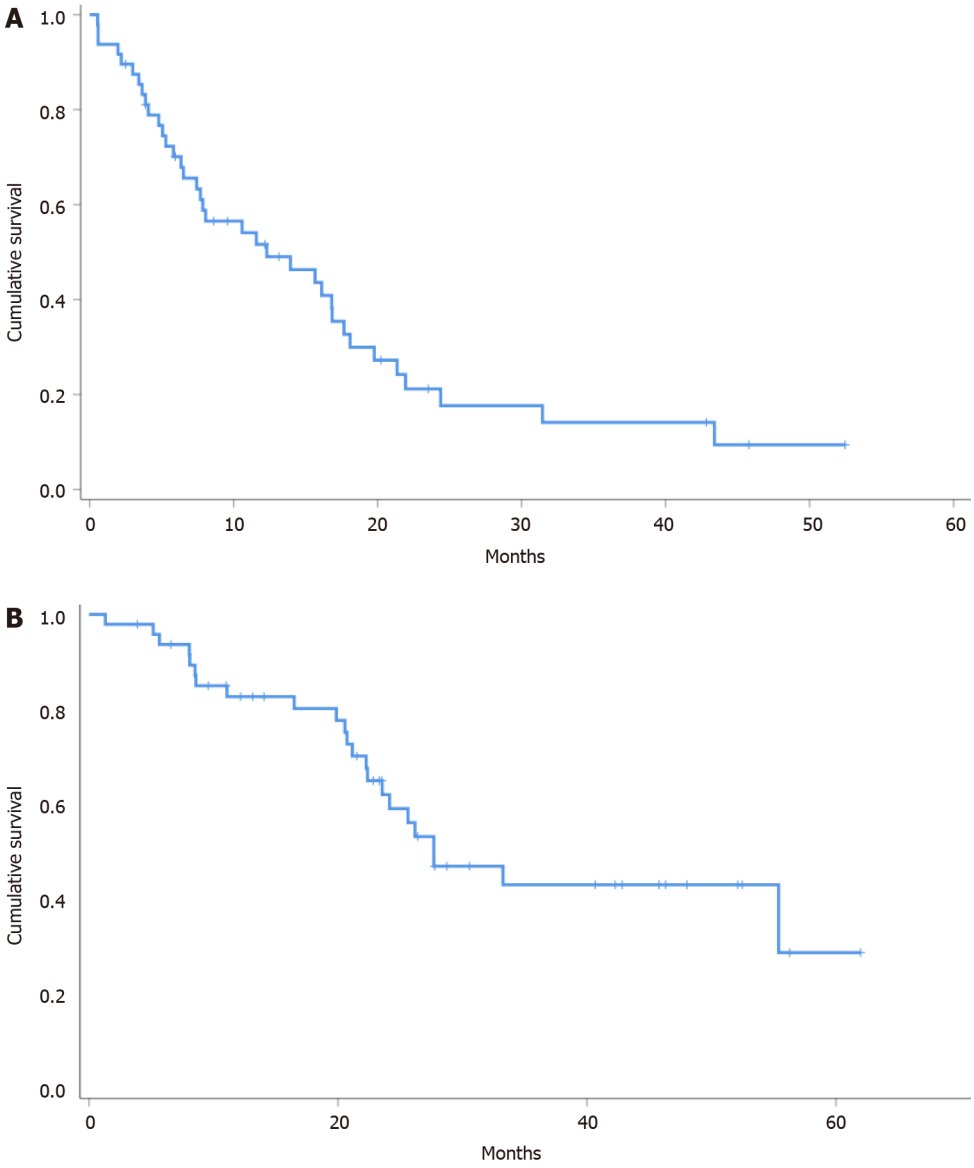


Figure 1 Progression-free survival (A) and overall survival (B) of the study population.

exclusive immunotherapy strategy (NCT03075423).

CONCLUSION

On the one hand, RCC with sarcomatoid features (irrespective of the primary histology) indeed gains excellent benefit from immunotherapy compared to TKIs[18]. On the other hand, RCC with variant histology still needs a TKI-based approach, with new generation TKIs, possibly combined with ICIs in future trials.

Meanwhile, the single-agent options should include pazopanib, favored by excellent manageability in terms of safety, and supported by high feasibility and excellent cost-effectiveness, especially for elderly patients.

ARTICLE HIGHLIGHTS

Research background

Non-clear cell metastatic renal-cell carcinoma (nccRCC) has dismal results with standard systemic therapies and a generally worse prognosis when compared to its clear-cell counterpart.

Research motivation
We aimed to provide a piece of real-life evidence on the use of pazopanib in this patient subgroup.

Research objectives
To assess the activity, efficacy, and safety of pazopanib as first-line therapy for advanced nccRCC patients treated in a real-life setting.

Research methods
This was a multicenter retrospective observational analysis.

Research results
The objective response rate with pazopanib was 27.1%, the disease control rate was 83.3%, and the median progression-free survival and overall survival were 12.3 (95%CI: 3.6-20.9) mo and 27.7 (95%CI: 18.2-37.1) mo, respectively. Grade 3 adverse events occurred in 20% of patients, and no grade 4 or 5 toxicities were found.

Research conclusions
Pazopanib should be considered as a good first-line option for metastatic RCC with variant histology.

Research perspectives
Pazopanib warrants further development in metastatic RCC with variant histology.

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ORIGINAL ARTICLE

Retrospective Study

Pathological response to neoadjuvant therapy with chemotherapy *vs* chemoradiotherapy in stage III NSCLC-contribution of IASLC recommendations

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Hospital Clínic de Barcelona.

Conflict-of-interest statement: We have no conflict of interest or financial relationships to disclose.

Data sharing statement: No additional data are available.

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Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): 0
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Abstract

BACKGROUND

Neoadjuvant treatment (NT) with chemotherapy (Ch) is a standard option for resectable stage III (N2) NSCLC. Several studies have suggested benefits with the addition of radiotherapy (RT) to NT Ch. The International Association for the Study of Lung Cancer (IASLC) published recommendations for the pathological response (PHR) of NSCLC resection specimens after NT.

AIM

To contribute to the IASLC recommendations showing our results of PHR to NT Ch *vs* NT chemoradiotherapy (ChRT).

METHODS

We analyzed 67 consecutive patients with resectable stage III NSCLC with positive mediastinal nodes treated with surgery after NT Ch or NT ChRT between 2013 and 2020. After NT, all patients were evaluated for radiological response (RR) according to Response Evaluation Criteria in Solid Tumours criteria and evaluated for surgery by a specialized group of thoracic surgeons. All histological samples were examined by the same two pathologists. PHR was evaluated by the percentage of viable cells in the tumor and the resected lymph nodes.

RESULTS

Forty patients underwent NT ChRT and 27 NT Ch. Fifty-six (83.6%) patients underwent surgery (35 ChRT and 21 Ch). The median time from ChRT to surgery was 6 wk (3-19) and 8 wk (3-21) for Ch patients. We observed significant differences in RR, with disease progression in 2.5% and 14.8% of patients with ChRT and Ch, respectively, and partial response in 62.5% ChRT *vs* 29.6% Ch (P = 0.025). In PHR we observed ≤ 10% viable cells in the tumor in 19 (54.4%) and 2 cases (9.5%), and in the resected lymph nodes (RLN) 30 (85.7%) and 7 (33.3%) in ChRT and Ch, respectively (P = 0.001). Downstaging was greater in the ChRT compared to the Ch group (80% *vs* 33.3%; P = 0.002). In the univariate analysis, NT ChRT had a significant impact on partial RR [odds ratio (OR) 12.5; 95% confidence interval (CI): 1.21 - 128.61; P = 0.034], a decreased risk of persistence of cancer cells in the tumor and RLN and an 87.5% increased probability for achieving downstaging (OR 8; 95%CI: 2.34-27.32; P = 0.001).

CONCLUSION

We found significant benefits in RR and PHR by adding RT to Ch as NT. A longer follow-up is necessary to assess the impact on clinical outcomes.

Key Words: Non-small cell lung cancer; Chemotherapy; Chemoradiotherapy; Neoadjuvant treatment; Resectable stage III; Pathological response

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Core Tip: Preoperative chemotherapy (Ch) has become a standard treatment option, especially in resectable stage III (primarily N2) non-small cell lung cancer (NSCLC). Phase II and phase III studies have raised the question as to whether preoperative Ch plus radiotherapy provides any additional benefits to preoperative Ch. The objective of our retrospective study was to contribute (with an experienced team of medical oncologists, radiation oncologists, thoracic surgeons, and pathologists) to the International Association for the Study of Lung Cancer recommendations in relation to

differences in the pathological evaluation of tumors and mediastinal and hilar nodes in resectable stage III NSCLC, comparing neoadjuvant Ch *vs* chemoradiotherapy.

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INTRODUCTION

The International Association for the Study of Lung Cancer (IASLC) recently published recommendations for the pathological evaluation of histological specimens from the resection of non-small cell lung cancer (NSCLC) after neoadjuvant therapy (NT)[1]. This article describes the lack of consensus in clinical practice regarding the evaluation of surgical specimens and in precise definitions of the degree of pathological response (PHR).

The seminal articles by both Junker *et al*[2] and Pataer *et al*[3] have been the main guide for the pathological evaluation of NT in recent decades. Although Junker *et al*[2] proposed that the lymph nodes of these patients be evaluated in the same way as the primary tumor (pT), the importance of the precise histological characteristics of these nodes has not been well established.

In relation to treatment, NT chemotherapy (Ch) has become a standard treatment option, especially in resectable stage III NSCLC[4]. However, later phase II and phase III studies raised the question as to whether NT Ch plus radiotherapy (RT) provides any additional benefit to NT Ch[5,6].

In 1996, the Lung Cancer Committee of our hospital (LCCHCB) initiated the first phase II trial of induction chemoradiotherapy (ChRT) in stage III NSCLC in Spain. We used the histological evaluation recommended by Junker *et al*[2] to describe the percentage of viable cells in resected neoplastic samples. The long follow-up of these results was presented at several international congresses[7].

In 2012, following the results of the Intergroup phase III trial[8], the LCCHCB and three other hospitals developed a new phase II trial with NT in resectable stage III NSCLC patients with exclusive lobectomy and a histologically proven single mediastinal lymph node level. In the Hospital Clínic de Barcelona (HCB), induction "per protocol" was with radical ChRT up to 60 Gy, and in the other hospitals the induction was performed with Ch alone (except for Pancoast tumors that also received induction of ChRT). After NT, all the patients were evaluated for radiological response (RR) according to Response Evaluation *Criteria* in Solid Tumours (RECIST) criteria and for surgery by a specialized group of thoracic surgeons. All the histological samples were examined by the same two pathologists.

The objective of this retrospective study was to contribute (with an experienced team of medical oncologists, radiation oncologists, thoracic surgeons, and pathologists) to the IASLC recommendations on differences in the pathological evaluation of tumor and mediastinal and hilar nodes in resectable stage III NSCLC comparing NT Ch *vs* ChRT.

MATERIALS AND METHODS

Patient selection

The population studied in this retrospective analysis were all patients consecutively diagnosed with resectable stage III NSCLC (AJCC, 8th edition) with pathologically proven single positive mediastinal lymph nodes treated between 2013 and 2020. NT treatment was followed by surgery with intention-to-cure according to the protocols of the four participating institutions and the decision of the respective Lung Cancer Committees. Some patients with N3 Level involvement and stage IV (single brain

metastasis previously treated with radiosurgery) were accepted for salvage thoracic surgery by decision of the LCCHCB. Patients with histologically demonstrated NSCLC but N2 Lymph nodes not accessible for biopsy were eligible, provided that the N2 node had a diameter greater than 1 cm and was positron emission tomography/computerized tomography (PET/CT) positive.

Patients who did not fully complete the curative treatment course were included in the study for intention-to-treat analysis.

Pre-treatment evaluation

All patients were evaluated by the multidisciplinary Lung Cancer Committee in each hospital which was composed of pulmonologists, radiologists, nuclear radiologists, medical oncologists, radiation oncologists and thoracic surgeons. The initial diagnostic study included radiological evaluation by chest CT, PET/CT, brain imaging [CT or brain magnetic resonance imaging (MRI)], and cardiopulmonary tests. Mediastinal and hilar involvement was confirmed by mediastinoscopy, endobronchial ultrasound (EBUS) and/or esophageal ultrasound (EUS). Before surgery, all patients were reassessed by the committee using chest CT to assess response to NT, and patients not demonstrating progression were considered for thoracic surgery.

Treatment

NT with ChRT or Ch was performed according to the protocol of each hospital and according to the clinical characteristics of each patient. All the patients included were considered for radical surgery by resection of the tumor and extensive resection of the hilar and mediastinal lymph nodes. RT was performed in the ChRT group concomitant with Ch, using 3 dimensional (D) conformal RT with standard fractionation of 2 Gy per fraction up to a total dose of 60 Gy (range 58-62 Gy). RT was administered to the pT and affected lymph nodes, with margins for microscopic disease and patient set up error. 4D assessment was not used, and involved-field of tumor and nodal treatment was exclusively adopted. Patients in both groups received platinum-based Ch without consolidation Ch after surgical resection. In the NT-Ch group, patients with R1 resection or persistence of viable cells in N2 Lymphadenectomy received postoperative RT (PORT) up to 54-60 Gy.

Post-NT evaluation and treatment

Chest CT scan was used to assess RR between 3 wk and 4 wk after NT according to the RECIST criteria. Operable patients who did not progress during NT were considered for radical surgical treatment. All patients were operated centrally in the same hospital (HCB). After surgery, PHR was evaluated by the same team of pathologists. Patients not considered for surgery after NT were treated with radical ChRT.

Histological assessment of pT and lymph nodes

PHR was defined according to the percentage of viable cells in the sample in both the pT and in the pathological lymph nodes (pN). It was categorized into five groups: 0%-10%; 11%-30%; 31%-50%, 51%-70%; > 70%, according to the literature. pN were taken into account to determine the downstaging rate.

Small volume samples (up to 2 cm) of pT and pN were completely sampled. In larger tumors, at least one paraffin block per cm of the largest diameter of the tumor was sampled in each specimen. The percentages of viable tumor cells, tumor necrosis, and fibrosis were evaluated in each slide, and the average of the percentages of viable tumor cells was reported for each patient. In specimens evaluated during 2020, PHR was evaluated following the IASCL recommendations[1]. In these cases, an entire cross-section of the tumor bed was sampled and photographed matching the areas on the specimen corresponding to the submitted blocks, and the percentage of necrosis, stromal tissue and viable tumor of the tumor bed was recorded. The components of the stromal tissue, fibrosis and inflammation were not specified.

Statistical analysis

Data were retrospectively analyzed after previous approval by the institutional review board. Two cohorts of patients were analyzed: NT ChRT plus surgery and NT Ch plus surgery. The parameters analyzed and compared in each group were: Mean age, sex, performance status, stage at diagnosis, TNM description, lung tumor location, N2-N3 confirmation, pathological distribution of the affected lymph node level, Ch scheme based on platinum doublet, acute RT or Ch toxicity, RR according to RECIST, and the median time from the end of NT to surgery. The type of surgery and complications of the intervention, levels of systematic lymph node dissection, the presence of DS, the

percentage of histological tumor viability (HTV) and lymph node histological viability (HLNV) were also analyzed. Both HTV and HLNV were also analyzed according to the histological type (adenocarcinoma *vs* squamous carcinoma) and NT type (ChRT *vs* Ch).

We performed a descriptive analysis comparing variables related to demographic, clinical, treatment and response data. The student's *t* test was used for quantitative variables, while the chi-square and Fisher exact test were applied for qualitative variables. Univariate analysis was carried out to assess the impact of ChRT on response using a logistic regression model. The statistical analyses were performed by a radiation oncologist with expertise in the IBM SPSS© version 25.0.

RESULTS

Between 2013 and 2020, 67 patients with resectable locally advanced NSCLC were evaluated (66 stage III and one stage IV with single brain metastasis, previously treated with radiosurgery); 65% were men, and the median age was 64 years (41-79). More than 90% of the patients had good functional status (ECOG PS ≤ 1). The most frequent histology was adenocarcinoma (62.5% ChRT and 74.1% Ch). Pathological nodal confirmation at diagnosis was made in most patients (87.5% ChRT and 92.6% Ch) by EBUS/EUS, mediastinoscopy or both. The clinical characteristics of the patients are summarized in Table 1.

In the ChRT group 47.5% of the patients simultaneously received Ch with cisplatin plus VP16 *vs* 0% in the Ch group, while 37% of the Ch group received the carboplatin-based doublet (with vinorelbine, taxol or gemcitabine) *vs* 7.5% in ChRT (with vinorelbine) (Table 2). Of the 40 ChRT patients, 28 (70%) started concomitant treatment within less than 7 d, 9 (22.5%) patients started at between 7 d and 14 d, and in 3 (7.5%) concomitant treatment was initiated after more than 14 d.

A total of 10 patients required hospitalization secondary to NT; 8 (20%) in the ChRT group *vs* 2 (7.4%) in the Ch group. Grade 2-3 esophagitis was observed in 10 (14.5%) patients with ChRT (only 2 grade 3) compared with no esophagitis in the Ch group.

Five patients in the ChRT group presented toxicity after surgery; 2 presented atrial fibrillation that was treated pharmacologically, 2 cases required surgical management due to bronchio-pleural fistula and cerebrospinal fluid fistula, and one patient presented grade 5 toxicity due to complicated bronchio-pleural fistula five months after surgery. No case of surgical toxicity was observed in the Ch group.

The RR showed a statistically significant difference (P = 0.025) in favor of NT with ChRT, with no case of local progression.

One patient (2.5%) in the ChRT group presented disease progression with brain metastases prior to surgery *vs* 4 patients (14.8%) in the Ch group who presented local progression during Ch.

Twenty-five (62.5%) ChRT patients showed partial RR compared to 8 (29.6%) in the Ch group (Table 3).

A total of 11 patients did not undergo surgery after committee reassessment. Of these, 5 (12.5%) were in the ChRT group, and surgery was not performed due to poor post-NT respiratory functionalism in 1, high risk of pneumonectomy in another, in 1 patient symptomatic brain metastasis was detected prior to surgery, 1 developed bilateral pneumonia secondary to influenza type A, and 1 patient presented poor respiratory functionalism prior to NT and stable radiological disease and suspicion of brain metastasis on MRI. The remaining 6 (22.2%) Ch patients did not undergo surgery: 1 for poor post-NT respiratory function and high risk of pneumonectomy, 3 for local disease progression during Ch, and 2 patients showed poor response after NT. The characteristics of the surgery according to NT group are shown in Table 4.

Lobectomy was the most frequent type of surgery (60% ChRT and 76% Ch), although this was more complex (due to vascular reconstruction, rib resection and / or vertebrectomy) in 25.7% *vs* 9.6% of patients receiving ChRT compared to Ch, respectively.

The median time from the end of NT with ChRT to surgery was 6 wk (3-19), and the median time from the end of the NT Ch to surgery was 8 wk (3-21).

In relation to PHR there was a statistically significant difference in favor of the ChRT group (Table 5). Maximum response (≤ 10% viable cells) was observed in the tumor in 54.5% *vs* 9.5% (P = 0.001) and at the lymph node level in 85.5% *vs* 33.3% (P = 0.001) with ChRT and Ch, respectively. Downstaging was achieved in 28 (80%) and 7 (33.3%) ChRT and Ch patients, respectively (P = 0.002). Two patients in the ChRT group showed lymph node involvement outside the RT field after surgery, but with

Table 1 Patient characteristics, *n* (%)

	RTCh (*n* = 40)	Ch (*n* = 27)	*P* value
Age	60 (54-67)	67 (62-73)	0.32
Gender			0.29
Male	26 (65)	21 (77.8)	
Performance status			0.21
ECOG 0	7 (17.5)	5 (18.5)	
ECOG 1	33 (82.5)	20 (74.1)	
ECOG 2	0	2 (7.4)	
Smoking habit			0.48
Yes	20 (50)	15 (55.6)	
No	2 (5)	3 (11.1)	
Former smoker	18 (45)	9 (33.3)	
Tumor localization			0.33
Apex	7 (17.5)	1 (3.7)	
Right upper lobe	13 (32.5)	13 (48.1)	
Right lower lobe	6 (15)	2 (7.4)	
Left upper lobe	12 (30)	9 (33.3)	
Left lower lobe	2 (5)	2 (7.4)	
Histology			0.28
Adenocarcinoma	25 (62.5)	20 (74.1)	
Squamous	13 (32.5)	6 (22.2)	
NSCLC[1]	0	1 (3.7)	
Large cell	2 (5)	0	
Stage			0.52
IIIA	27 (67.5)	21 (77.8)	
IIIB	12 (30)	6 (22.2)	
IV	1 (2.5)	0	
T			0.08
T1	4 (10)	9 (33.3)	
T2	16 (40)	9 (33.3)	
T3	10 (25)	7 (25.9)	
T4	10 (25)	2 (7.4)	
N			0.04
0	5 (12.5)	0	
2	34 (85)	27 (100)	
3	1 (2.5)	0	
Metastasis	1 (2.5)	0	0.59
Nodal station distribution			0.012
N0	6 (15)	0	
1N2	12 (30)	19 (70.4)	
2N2	2 (5)	1 (3.7)	

1N2 + 1N1	13 (32.5)	7 (25.9)	
2N2 + 1N1	5 (12.5)	0	
1N1 + 1N2 + 1N3	2 (5)	0	
Nodal staging method			0.68
EBUS	24 (60)	14 (51.9)	
Mediastinoscopy	2 (5)	3 (11.1)	
EBUS and mediastinoscopy	8 (20)	6 (22.2)	
EUS	1 (2.5)	2 (7.4)	
None	5 (12.5)	2 (7.4)	

[1]Specific histology could not be determined in one patient. Ch: Chemotherapy. ECOG: Eastern Cooperative Group. NSCLC: Non-Small Cell Lung Cancer. RTCh: Radiochemotherapy; EUS: Endoscopic ultrasound; EBUS: Endobronchial ultrasound

Table 2 Chemotherapy regimens, *n* (%)

	RTCh	Ch
	(*n* = 40)	(*n* = 27)
Cisplatin-Etoposide	19 (47.5)	0
Cisplatin-Vinorelbine	14 (35)	6 (22.2)
Cisplatin-Pemetrexed	4 (10)	3 (11.1)
Cisplatin-Docetaxel	0	6 (22.2)
Cisplatin-Gemcitabine	0	2 (7.4)
Carboplatin-Vinorelbine	3 (7.5)	1 (3.7)
Carboplatin-Paclitaxel	0	6 (22.2)
Carboplatin-Pemetrexed	0	1 (3.7)
Carboplatin-Gemcitabine	0	2 (7.4)

Ch: Chemotherapy. RTCh: Radiochemotherapy.

Table 3 Radiological response, *n* (%)

	RTCh	Ch	*P* value
	(*n* = 40)	(*n* = 27)	
Disease progression	1 (2.5)	4 (14.8)	0.025
Stable disease	14 (35)	14 (51.9)	
Partial response	25 (62.5)	8 (29.6)	
Complete response	0	1 (3.7)	

Ch: Chemotherapy. RTCh: Radiochemotherapy.

maximum PHR in the nodes within the RT field. Numerical but not significant differences were found in complete PHR (17.1% ChRT *vs* 4.8% Ch; $P = 0.23$).

In addition, the Ch group had a higher rate of "non-response" to NT in the tumor (> 70% viable cells pT) 38.1% *vs* 2.9% for ChRT ($P = 0.001$), being 42.9% Ch *vs* 5.7% ChRT ($P = 0.001$) at the lymph node level (> 70% viable pN cells).

In the univariate analysis, the use of NT ChRT had a significant impact on the RR with an increased probability of presenting partial response [odds ratio (OR) 12.5; 95% confidence interval (CI): 1.21-128.61; $P = 0.034$]. In addition, NT ChRT patients presented a decreased risk of persistence of cancer cells in the tumor and resected

Table 4 Surgery characteristics, *n* (%)

	RTCh (*n* = 40)	Ch (*n* = 27)	*P* value
No surgery	5 (12.5)	6 (22.2)	
Type of surgery			0.48
Lobectomy	21 (60)	16 (76)	
Bilobectomy	3 (8.6)	1 (4.8)	
Pneumonectomy	2 (5.7)	1 (4.8)	
Lobectomy and vascular reconstruction	1 (2.8)	1 (4.8)	
Lobectomy and rib resection	5 (14.3)	1 (4.8)	
Lobectomy with rib resection and vertebrectomy	3 (8.6)	0	
Segmentectomy with rib resection and vertebrectomy	0	1 (4.8)	
Node level dissection			0.26
2N2 + 1N1	4 (11.4)	3 (14.3)	
3N2	2 (5.7)	5 (23.7)	
3N2 + 1N1	12 (34.3)	7 (33.3)	
3N2 + 2N1	5 (14.3)	3 (14.3)	
4N2 + 1N1	8 (22.9)	1 (4.8)	
4N2 + 2N1	0	1 (4.8)	
4N2 + 1N3	2 (5.7)	0	
5N2 + 1N1	2 (5.7)	1 (4.8)	

Ch: Chemotherapy. RTCh: Radiochemotherapy.

lymph nodes (Table 6). Patients in the ChRT group presented an 87.5% increased probability of presenting lymph node downstaging (OR 8; 95%CI: 2.34-27.32; P = 0.001).

When analyzing both HTV and HLNV by histological (adenocarcinoma *vs* squamous carcinoma) and NT type, remarkable results were not obtained due to the limited number of patients in each group.

DISCUSSION

In this retrospective multi-institutional phase II study we compared NT Ch *vs* NT ChRT and present the RR and PHR following NT in patients with stage III NSCLC treated by lung cancer specialists from 4 experienced university centers.

Several randomized clinical trials (RCT) have compared NT Ch to NT ChRT, but the results of some of these studies have only been reported in abstract form[9,10], which precludes a real scientific analysis, especially at the level of PHR. Another phase II RCT was published comparing NT Ch *vs* NT ChRT[11], but no relevant information was obtained due to the small number of patients included (n = 46) which were further divided into three different groups. In addition, the main endpoint of this study was the feasibility of surgery, and PHR results were not provided.

Thomas *et al*[5] published a RCT that compared a control group undergoing induction Ch with 3 cycles of cisplatin and VP16 followed by surgery and PORT *vs* an intervention group that underwent NT Ch with the same regimen followed by twice-daily RT (45 Gy) concomitant with carboplatin and vindesine, followed by surgery. In the study, 54% and 59% of ChRT and Ch patients underwent surgery, respectively, and only 37% and 32% of each group, respectively achieved complete resection. This may be due to the inclusion of a proportion of locally advanced NSCLC not clearly resectable at first (15% with T4N2 and 22% with T4N3). It is also important to note that about 20% of the patients in each group progressed to induction Ch prior to NT ChRT

Table 5 Pathological response, *n* (%)

	RTCh (*n* = 40)	Ch (*n* = 27)	*P* value
No surgery	5 (12.5)	6 (22.2)	
Pathological complete response	6 (17.1)	1 (4.8)	0.23
Tumor response[1]			0.001
0%-10%	19 (54.4)	2 (9.5)	
11%-30%	9 (25.7)	2 (9.5)	
31%-50%	4 (11.4)	3 (14.3)	
51%-70%	2 (5.7)	6 (28.6)	
> 70%	1 (2.8)	8 (38.1)	
Nodal response[1]			0.001
0%-10%	30 (85.7)	7 (33.3)	
11%-30%	1 (2.8)	0	
31%-50%	2 (5.7)	3 (14.3)	
51%-70%	0	2 (9.5)	
> 70%	2 (5.7)	9 (42.9)	
Downstaging	28 (80)	7 (33.3)	0.002

[1]According to the percentage of viable cells in the histological study.
Ch: Chemotherapy. RTCh: Radiochemotherapy.

or surgery, and all patients with NT Ch received PORT.

Each endpoint favored NT ChRT with more complete resection (75% *vs* 60%; P = 0.008), nodal downstaging from N2 to N0-1 (46% *vs* 29%, P = 0.02) and PHR greater than 90% (60% *vs* 20%; $P < 0.0001$). Patients with complete resection and mediastinal downstaging presented a greater overall survival (OS), with no differences in progression-free survival (PFS) or OS in relation to the different types of NT.

Katakami *et al*[12] published an RCT of 60 pathologically proven N2 patients randomized to receive induction Ch with docetaxel and carboplatin plus concurrent RT (40 Gy) followed by surgery or NT Ch followed by surgery. Nodal downstaging was described in 20.8% and 40% in Ch and ChRT, respectively. The median OS in patients with and without downstaging in the ChRT arm was 72.1 mo and 31.2 mo, respectively (P = 0.018) and 32.6 mo and 29.0 mo, respectively (P = 0.542) in the Ch arm. The median PFS and OS in each study arm were not statistically significant due to the small sample size.

The next RCT comparing NT Ch *vs* NT ChRT was performed by Pless *et al*[13]. Similar to the trial by Thomas *et al*[5], this study was not a true and strict comparison between NT Ch and NT ChRT. Patients were randomized to 3 cycles of NT Ch (cisplatin and docetaxel) plus sequential RT *vs* NT Ch. All patients were scheduled for surgery. An additional difference with the Thomas trial was that PORT was only administered in the case of microscopic (R1) or macroscopic (R2) tumor margins (16% of trial patients). Patients treated with sequential NT ChRT presented higher lymph node downstaging (64% *vs* 53%), albeit without statistically significant differences. In this study, it is also important to point out two things. First, all the patients were resectable and had low-bulky disease, and second, the trimodal therapy was not radical NT with concurrent ChRT, but rather NT Ch plus sequential RT and surgery. Sequential ChRT is far from being as effective as concurrent ChRT. To our knowledge no study has compared the differences in PHR of sequential *vs* concurrent ChRT, but there is a meta-analysis on the efficacy of these treatments[14].

A true trimodality phase II trial was published by the RTOG 02-29[15]. This trial evaluated downstaging rates in 57 patients with stage III NSCLC (pathologically proven N2 or N3) who received weekly carboplatin and paclitaxel with concurrent RT (50.4 Gy to mediastinal nodes and pT and 10.8 Gy boost to gross disease). The mediastinum was pathologically reassessed after completion of ChRT. Forty-three

Table 6 Univariate analysis investigating the impact of neoadjuvant therapy radiochemotherapy on the response

	OR	95%CI	*P* value
Radiological response			
Disease progression	Reference		
Stable disease	4	0.39-40.42	0.240
Partial response	12.5	1.21-128.61	0.035
Complete response	NA[1]		
Pathological tumor response			
0%-10%	Reference		
11%-30%	0.474	0.05-3.92	0.489
31%-50%	0.14	0.17-1.13	0.065
51%-70%	0.03	0.004-0.30	0.002
> 70%	0.01	0.001-0.16	0.001
Pathological nodal response			
0%-10%	Reference		
11%-30%	0.474	0.057-3.92	0.489
31%-50%	0.14	0.17-0.13	0.065
51%-70%	0.03	0.004-0.30	0.002
> 70%	0.01	0.001-0.16	0.001
Downstaging			
No	Reference		
Yes	8	2.34-27.32	0.001

[1]Not applicable: only one patient in the chemoradiotherapy group and none in the Chemotherapy group. CI: Confidence interval.

patients (75%) were evaluable for the primary endpoint. Twenty-seven patients achieved the primary endpoint of downstaging (63%). Thirty-seven patients underwent resection. The 2-year OS rate was 75% for those who achieved downstaging, 52% for those with residual nodal disease, and 23% for those who were not evaluable for the primary endpoint ($P = 0.0002$).

Radical ChRT attempts to eliminate the possibility of administering a subtherapeutic dose of RT (45 Gy) and/or RT interruptions in patients who, for whatever reason, cannot undergo surgery. The rationale for combining NT Ch with full-dose RT was supported by a retrospective analysis of RT series in NSCLC in which a dose in the range of 58–77 Gy might be necessary to control 50% of gross tumors[16].

During the years of the RCTs comparing NT with Ch and ChRT in stage III NSCLC, other RCTs such as that of the EORTC 0894[17], INT 0139[8] and the ESPATUE trial [18], carried out another line of research on whether surgery adds any real benefits in OS compared to radical concurrent ChRT.

The EORTC 08941 RCT randomized patients to definitive RT *vs* surgery (with PORT in patients with R1 resection) in NSCLC with pathologically proven N2 responding to the initial Ch doublet. It should be noted that 39% of the 579 patients progressed or did not respond to NT Ch and only 61% of all patients were randomized to RT or surgery. There were no statistically significant differences in PFS or OS between the two groups.

In the INT 0139 trial, 396 patients with resectable stage IIIA N2 NSCLC were randomized to concurrent radical RT (61 Gy) with 2 cycles of cisplatin and VP16 *vs* concurrent RT (45 Gy) with the same Ch regimen and surgery. The median OS was not significantly improved with the addition of surgery. However, in an exploratory analysis, the median OS was significantly higher for patients undergoing lobectomy compared with definitive ChRT (33.6 *vs* 21.7 mo; $P = 0.002$). Other factors related to an improvement in OS were pathologic N0 status (34.4 *vs* 26.4 mo; $P \leq 0.0001$) and pathological complete response (PCR) (T0N0: 39.8 mo). Criticisms regarding the INT

0139 trial were the incomplete accrual rate, an under-powered subset analysis suggesting an advantage of trimodal therapy, and a very high mortality rate among patients who underwent pneumonectomy[19]. It should also be noted that the EORTC and the Intergroup trials were designed at a time when routine PET/CT scan had not yet been incorporated into usual clinical practice, and nodal staging using EBUS or EUS was not available in most hospitals.

The ESPATUE trial compared definitive treatment with ChRT *vs* trimodal therapy. Patients with stage IIIA (N2) and IIIB NSCLC underwent 3 cycles of NT Ch with cisplatin and paclitaxel. Patients who did not progress were treated with hyperfractionated RT (45 Gy in 30 fractions twice daily) plus Ch. The patients were reevaluated for operability during the last week of RT, and those eligible for surgery were randomized to completing RT or surgery. The study closed prematurely, with 246 patients being enrolled and 161 patients randomized. Seventy of 81 of the surgical patients underwent surgery, of which 66 underwent R0 resection. A total of 5 (7%) patients presented grade 5 toxicity after surgery. After a median follow-up of 78 mo, there were no differences in PFS or OS.

A cumulative meta-analysis of RCT comparing definitive ChRT *vs* NT therapy followed by surgery[20] in stage III NSCLC found no significant differences in OS in these patients after NT Ch and surgery. It is also noteworthy that the trials with concurrent NT ChRT and radical ChRT showed a better OS than the other trials with NT Ch alone before surgery.

Another line of research has been to combine ChRT with antibodies against epidermal growth factor receptors (EGFR) in stage III NSCLC. The NRG oncology RTOG 0839 study[21] was designed to test the hypothesis that adding an EGFR antibody to the standard ChRT could potentially improve the outcome in this group of patients. The endpoint was downstaging, but an unexpectedly high mortality rate was observed in the panitumumab group.

In addition, a phase II RCT with erlotinib *vs* gemcitabine plus cisplatin as NT was performed in patients with stage IIIA-N2 NSCLC with EGFR mutations in exon 19 or 21[22]. A total of 72 patients were randomized. No PCR was found in any of the arms. Three of 31 patients in the erlotinib arm and none of 23 Ch patients achieved maximal pathologic response (MPR).

A recent line of investigation of NT in stage III (N2) NSCLC has been the combination of concomitant Ch with immunotherapy. NADIM, a single-arm, phase II multicenter trial in resectable stage IIIA NSCLC, included 46 patients who received 3 cycles of NT Ch plus nivolumab followed by surgical resection, and then continued nivolumab for one year. At 24 mo, PFS was 77.1% (95%CI: 59.9-87.7). No patient presented disease progression during NT, and 34 (83%; 95%CI: 68-93) of 41 operated patients had MPR, 26 of whom (63%; 95%CI: 62-91) showed PCR. Thirty-seven (90%) of the 41 patients operated presented nodal downstaging (from N2 to N1-N0)[23].

For nearly three decades, PHR after NT has been correlated with survival[24]. According to Pisters *et al*[24] PCR predicts a higher OS, which is considered an important endpoint for Ch. However, the mean frequency of PCR after NT Ch is 4% to 7% (the PCR rate appears to be higher in squamous cell carcinomas). Due to the low proportion of PCR after NT Ch, another surrogate parameter of OS in relation to PHR was considered for NT Ch by Hellman *et al*[25] who proposed MPR, defined as a value < 10 % of viable cells in resected tumors, as a surrogate endpoint for OS. This proposal was based on a previous analysis by Pataer *et al*[3] in 192 resected stage I-IV NSCLC patients treated with NT Ch. The percentage of viable tumor cells after NT Ch was considered a categorical variable and analyzed in relation to the risk of death. A significant improvement in OS was demonstrated in those with 0-10% viable tumor cells compared to other groups (11%-30%, 31%-50%, 51%-70%, and 71%-100%).

This correlation between MPR and OS has not been validated in NT ChRT[5], although a positive association between lymph node downstaging and improved OS has been shown in pathological IIIA N2 NSCLC after NT ChRT and NT Ch[25]. Even if the degree of PHR is a predictor of OS, only a few studies have described a detailed pathological assessment (percentage of viable cells) at the pT and metastatic node level. The PHR to NT can significantly vary between the tumor and metastatic lymph nodes. The extent of lymphadenectomy may vary between centers and may depend on the methodological approach of the surgeons and pathologists at each hospital. Furthermore, the minimum number of lymph nodes or lymph node levels that must be resected has not been defined. This last fact is directly related to the experience of thoracic surgeons in tumor resection and / or in the extension of radical complementary lymphadenectomy with a minimum of nodal stations evaluated.

There is little consensus on the precise definition of what is considered as "resectable" T4 or N2-N3 disease, and there is no universally accepted definition of "potentially resectable N2" disease. Surgical treatment largely depends on the experience of the hospital and the expertise of the thoracic surgeons involved. T4 disease involves a locally aggressive tumor with invasion of nearby mediastinal structures, such as the carina, great vessels, and / or vertebrae. In these cases, it can be very difficult to achieve complete resection (R0) as defined by the IASLC[26].

The importance of the so-called "time window" recommended for performing surgery after NT ChRT has been reported. A retrospective study of 1623 patients with stage IIIA NSCLC treated with concurrent NT ChRT found a statistically significant decrease in OS when surgery was performed more than 6 wk after completion of RT [27]. OS was compared in patients operated at 0-3 wk, 3-6 wk, 6-9 wk and 9-12 wk after completing RT. The multivariate analysis demonstrated no significant difference in those who underwent surgery within 6 wk of NT ChRT. However, significant reductions in OS were observed in patients operated at more than 6 wk and ≤ 9 wk after NT [hazard ratio (HR) = 1.33, 95%CI: 1.01-1.76, P = 0.043].

The optimal treatment strategy in resectable stage III (N2) NSCLC is debated, and different guidelines from the United Kingdom, Europe and the United States have made recommendations. The common recommendation is to administer multimodal treatment to prevent distant disease with systemic therapy and achieve local control by surgery, RT, or both. Furthermore, multimodal treatments require experienced multidisciplinary teams to minimize the secondary risks of the treatments and maximize their benefits[28].

The most recent guidelines are the National Institute for Health and Care Excellence guidelines[29]. On pages 33-34 of these guidelines, detailed recommendations for operable stage IIIA-N2 NSCLC are described: "... the available evidence showed that ChRT and surgery are more effective than ChRT alone in people well enough for surgery and when the disease is operable...There was an 89% chance that ChRT and surgery improved the mean OS time compared to ChRT...".

The authors of this recent English guideline do not recognize as methodologically true or scientific some meta-analyses and/or systematic reviews of different articles of supposed NT comparisons reporting the absence of benefits in OS with the addition of RT to Ch as NT in stage III (N2) NSCLC[30-34].

Our phase II study is the first study in the scientific literature on NT in patients with stage III NSCLC, in which a true comparison is made between NT ChRT *vs* NT Ch prior to surgery performed by specialists with lengthy experience and a centralized group of thoracic surgeons and pathologists. This latter fact avoids biases due to different surgical skills and reduces the probability of morbidity and mortality in stage III patients after NT[35]. The evidence supports the association between the experience of thoracic surgeons and lower mortality and improvement in long-term OS after lung resection[36]. In addition, histological evaluation by the same two pathologists with experience in lung cancer reduces interobserver variability, achieving greater homogeneity in the results. To our knowledge, this is the first study in the literature in which the same two pathologists made an exhaustive description of the percentage of viable cells in the tumor and in all lymph nodes resected after NT, actually comparing the response to Ch *vs* ChRT. Two other factors of our study must be highlighted. A detailed description of the lymph node stations (mediastinal and hilar) affected prior to NT was made as well as the lymph node levels resected by lymphadenectomy. To our knowledge, this has never been reported in any other NT study, and both could be considered as a factor of surgical quality and may have a positive prognostic role in the evolution of the disease. This pathological description of the resected nodes is being studied in order to better differentiate surgically treated patients into different stages[37].

The main limitation of our study is that it is not an RCT. However, there were no significant differences in the characteristics of the patients, such as gender, performance status, tumor location or histology (Table 1). Nevertheless, the median age was slightly higher in the NT Ch group compared to the NT ChRT group (67 *vs* 60 years), and we also observed a higher proportion of apical / Pancoast tumors (7 *vs* 1) and stages IIIB (16 *vs* 7) in the NT ChRT group compared to the NT Ch patients. There was a significant difference in the Ch schemes used (Table 2) in NT ChRT compared to NT Ch, with 55.6% of ChRT patients being treated with cisplatin-VP16 compared to 0% in the NT Ch group. There is no evidence in the literature that one cisplatin doublet-based regimen is better than another. There is perhaps more experience with concurrent cisplatin-VP16 treatment with RT, and therefore the data could support this scheme in favor to others[19].

Furthermore, in relation to the evaluation of the RR, it should be noted that 4 NT Ch patients (14.8%) progressed locally to NT compared to none in the NT ChRT group. Of these patients who progressed, 2 did not undergo surgery and the other 2 were considered by the Lung Committee for salvage surgery. Both underwent R1 resection, and were therefore considered for PORT, and 1 developed multiple metastases during PORT planning. This evolution with local progression of the tumor during Ch is consistent with the results (between 20% and 30%) of the previously commented studies of induction Ch (EORTC, ESPATUE and the study of Thomas *et al*[5]). In relation to PHR, more than 38% of the NT Ch patients did not respond to Ch, with persistence of 70% or more of viable cells in the tumor and/or the nodes. In contrast, local progression and unresponsiveness after concurrent NT ChRT was 0% and 2.8%, respectively, being very similar to NT with Ch and immunotherapy in the NADIM trial. Indeed, the limited response to NT Ch in approximately 50% of the patients has not been adequately addressed in any previous study to assess whether this could be detrimental to long-term outcomes, and studies are needed to determine the impact of this treatment on PFS and OS.

Regarding the type of surgery, we observed more complex interventions in the NT ChRT group, according to the more advanced stages (*e.g.*, T4) in 27.5% compared to 7.4% in the NT Ch group. The first group included patients with locally advanced NSCLC, which was not easily amenable to primary resection, and in whom treatment with definitive ChRT could have been a valid option. These patients could be stratified into high-volume nodal disease (bulky, multilevel N2 or N3) or locally invasive pT (T4 N0-1 or superior sulcus tumor). According to the literature, in these patients only the experience of the thoracic surgeon provides greater local control and perhaps greater PFS and OS compared to definitive ChRT[19]. In our opinion, locally advanced tumors were the main cause of greater post-surgical morbidity and complications in the NT ChRT (3) compared to NT Ch (0), with two brocho-pleural fistulas and one cerebrospinal fluid fistula. The last case was a Pancoast tumor treated with lobectomy and vertebrectomy. The remaining NT ChRT patients and all the NT Ch patients could be considered as low volume N2 disease (IIIA) in which total resection could be achieved.

The median time between the end of RT and surgery was 6 wk (3-19). This is slightly longer than the recommended time of 3 w to 4 wk for resection after NT ChRT [27]. The median time between the last cycle of Ch and surgery was 8 wk (3-21). The recommended time between NT Ch and the intervention is unknown, which may explain why our treatment interval was so long. Cases with a longer interval (*e.g.*, 19 wk and 21 wk) were patients requiring salvage surgery.

There was also a significant difference in favor of the NT ChRT group in the PHR in the tumor and lymph nodes (Table 5). It should be noted that the NT Ch patients obtained 4.8% PCR at the tumor level, which is in line with what was observed by Pataer *et al*[3].

In the analysis of lymph node downstaging, we found significant differences between the patients receiving NT ChRT and those with NT Ch. In the ChRT group, 2 patients had positive nodes outside the initially diagnosed nodes. This is probably due to the volume of radiation treatment in these patients being limited to gross disease, and thus, these "out-of-field" nodes did not receive RT, and no local effect should be expected at this level. The involvement of these nodes was limited to intracapsular microscopic disease, which was diagnosed in the detailed pathological study after radical lymphadenectomy.

When comparing the PHR of our results of the NT ChRT group with that of the NADIM trial (the study with the best results published to date in this type of patients), the results are slightly better in the NADIM[23] study, with MPR in the tumor of 83% and 90% downstaging compared to an MPR of 54.5% in the tumor and 80% downstaging in our NT ChRT group, and 9.5 % and 33.3% in the NT Ch group, respectively. A longer follow-up will show whether these differences in PHR between NT ChRT and NT Ch have any impact on the PFS and OS of our patients.

When most of our patients were treated, the interim results of the LungART trial on the role of PORT had not yet been published[38]. Our NT Ch patients with persistent pN2 after surgery received PORT (54 Gy) following the LungART recommendations.

After the results published by the NADIM[23] trial, it is unlikely that an RCT will be conducted comparing NT Ch *vs* NT ChRT with robust results. In our opinion, the current line of research that should be followed in patients with stage III NSCLC is the combination of NT with immunotherapy and Ch or RT, concomitantly or in consolidation, as in the PACIFIC study[39].

We therefore believe that despite not being a RCT or prospective study, the results of our study may be useful to guide NT in patients with resectable stage III (N2) NSCLC, according to the experience in multimodal treatment and the surgical skills of each center in this type of patient.

CONCLUSION

Neoadjuvant treatment with ChRT provides significant benefits in both radiological and PHR in patients with resectable stage III NSCLC. However, a longer follow-up is necessary to assess the impact on clinical outcomes.

ARTICLE HIGHLIGHTS

Research background

There is no standardized clinical consensus for the evaluation of the surgical specimen in patients with non-small cell lung cancer (NSCLC) who have received neoadjuvant treatment with chemotherapy, radiotherapy, or a combination of both.

Research motivation

Following the recently published recommendations by the International Association for the Study of Lung Cancer (IASLC) for the pathological evaluation of tumors and lymph nodes, we analyzed the radiological and pathological response of patients treated with neoadjuvant chemotherapy or chemoradiotherapy.

Research objectives

Our intention was to contribute to the IASLC recommendations with clinical results that reflect the differences in the response to neoadjuvant therapy with chemoradiotherapy *vs* chemotherapy.

Research methods

We performed a retrospective analysis of patients with resectable stage III NSCLC treated with chemotherapy or chemoradiotherapy as neoadjuvant treatment followed by surgery. All histological samples were examined to assess pathological response by the percentage of viable cells in the tumor and the resected lymph nodes.

Research results

We observed better results in the chemoradiotherapy group for both radiological and pathological response, with a lower risk of persistence of cancer cells in the tumor and resected lymph nodes, and with a greater probability of achieving downstaging.

Research conclusions

In this study we observed a greater response to neoadjuvant treatment when adding radiotherapy to chemotherapy. We believe that this could contribute to improving the management of this group of patients.

Research perspectives

Longer follow-up of these patients is necessary to establish a relationship between pathological response and clinical outcomes.

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ORIGINAL ARTICLE

Observational Study

Modulated electro-hyperthermia in stage III and IV pancreatic cancer: Results of an observational study on 158 patients

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Abstract

BACKGROUND

An increasing number of studies report the beneficial effects of regional hyperthermia in association with chemotherapy (CHT) and radiotherapy for the treatment of pancreatic cancer; in particular, the use of modulated electro-hyperthermia (mEHT) results in increased survival and tumor response.

AIM

To compare outcomes of CHT alone or in association with mEHT for the treatment of stage III and IV pancreatic cancer.

METHODS

This was an observational retrospective study; data were collected for patients

technical appendix, statistical code, and dataset are available.

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Grade B (Very good): B, B
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with stage III-IV pancreatic cancer that were treated with CHT alone or in combination with mEHT from 2003 to 2019. A total of 158 patients were included in the study out 270 patients screened in four Italian hospitals; 58 (37%) of these received CHT + mEHT and 100 (63%) CHT. CHT was mainly gemcitabine-based regimens in both groups.

RESULTS

Overall (19.5 mo *vs* 11.02 mo, $P < 0.001$) and progression-free (12 mo *vs* 3 mo, $P < 0.001$) survival were better for the CHT + mEHT group compared to the CHT group. The association of mEHT resulted also in an improvement of tumor response with disease control rate 95% *vs* 58% ($P < 0.001$) at 3 mo. Toxicity was comparable in the two study groups, and mEHT related adverse events were limited in 8 patients presenting G1-2 skin burns.

CONCLUSION

The addition of mEHT to systemic CHT improved overall and progression-free survival and local tumor control with comparable toxicity.

Key Words: Modulated electro-hyperthermia; Locally advanced pancreatic cancer; Tumor response; Survival

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Core Tip: Modulated electro-hyperthermia is a relatively new regional hyperthermia method. It targets tumor cell membranes and extracellular matrix to increase their temperature. New studies have appeared in tumor palliation reporting incremental benefits of chemotherapy and radiotherapy and few additional side effects. In patients with stage III and IV pancreatic cancer, modulated electro-hyperthermia in association with chemotherapy results in significant improvements of overall and progression-free survival and tumor response with comparable toxicity.

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INTRODUCTION

Pancreatic cancer has one of the worst prognoses in oncology, with a 5-year overall survival (OS) of 9%, and is the seventh most common cause of cancer deaths in the world in 2018[1]. Adenocarcinoma is the main morphology of this tumor (90%), and its incidence and mortality are increased during the last 2 decades[1]. Surgery followed by adjuvant therapy is the only curative treatment of pancreatic tumors; however, only 10%-20% of them are resectable at diagnosis, whereas 30%–40% are locally advanced and 50%-60% are metastatic.

Non-metastatic, locally advanced pancreatic cancer (LAPC) can be treated with neoadjuvant chemotherapy or chemoradiotherapy to allow surgical resection. Gemcitabine in association with nab-paclitaxel and FOLFIRINOX (leucovorin, fluorouracil, irinotecan, oxaliplatin) for fit patients are two current standard first-line options in LAPC, but they have high toxicity and often low efficacy[2-4]. Pancreatic cancer is quite resistant to radiotherapy (RT) and chemotherapy (CHT) because of its hypoxic microenvironment that diminishes sensitivity to these therapies. In order to increase tumor response, the use of regional hyperthermia is often associated to CHT and RT[5]. Regional hyperthermia (RHT) efficacy in cancer remission is well known, indeed, it enhances drug delivery and diffusion inside the tumor cells, improves blood flow, reduces hypoxia, and inhibits DNA repair, resulting in apoptosis[6].

RHT is achieved by increasing the cancer cells' temperature to 39.5-43 °C with an external device. A new method of RHT has been recently developed: The modulated

electro-hyperthermia (mEHT) that is performed with a 13.56 MHz capacitive coupled device. It targets malignant cell membranes and extracellular matrix, allowing to overcome the issue of reaching deep tumors and achieving homogenous heating[7,8]. The mEHT has comparable benefits to other RHT methods and improves survival and local tumor control for several tumors, including pancreatic cancer[9-11].

MATERIALS AND METHODS

Patient selection

This was a large retrospective observational multicentric study aimed to compare outcomes of chemotherapy alone or in association with mEHT for the treatment of locally advance pancreatic cancer, in terms of survival and tumor response. Data were collected retrospectively, and patients were selected according to the following inclusion criteria: > 18 years, stage III and IV pancreatic cancer, treatment with CHT alone or in combination with mEHT, and informed consent signed. Patients were excluded from the study if they had a pacemaker, bilirubin, or transaminase level > 3 times the normal value upper range level or bleeding.

From January 2003 to December 2019, 270 patients with stage III and IV pancreatic cancer were screened in four Italian hospitals; 158 of these patients met the inclusion criteria and were included in the study, 58 (37%) of these received CHT + mEHT and 100 (63%) CHT alone. CHT was mainly gemcitabine-based regimens in both groups.

mEHT protocol and device

Modulated electro-hyperthermia was performed using the EHY-2000plus device (CE0123, Oncotherm, Torisdorf, Germany), applying a radiofrequency current of 13.56 MHz as carrier frequency that was modulated by time-fractal fluctuation. The energy was transferred by capacitive coupling, with precise impedance matching[12].

The hyperthermia protocol included three mEHT treatments/week for 2 mo, starting at a 60 W power for 40 min. Following treatments were performed by increasing the power up to 150 W and the time up to 90 min in 2 wk. mEHT was administered after CHT or within 48 h, in order to couple the high drug blood concentration with the modulated electro hyperthermia and optimize their synergy.

The majority (95%) of gemcitabine-based treatments were administered on the same day of electro-hyperthermia treatment. In a minority of patients (5%), it was administered the following day or within the following 72 h because of precarious clinical conditions and geographic accessibility. Even if gemcitabine had a half-life of 42-94 min and was eliminated within 5-11 h after infusion, the pharmacokinetic elimination half-life for dFdU varies between 2 and 24 h, and it is still present systemically in concentrations greater than 1 μmol/L up to 1 wk after infusion[13].

Outcome measures

The primary outcome was to monitor OS and progression free survival (PFS). OS was considered from diagnosis to death or last follow up date; PFS was considered from treatment start to date of progression.

Secondary outcome was local tumor control and toxicity. Response Evaluation Criteria in Solid Tumors version 1.4 was used for tumor assessment from magnetic resonance imaging or computed tomography scans. Complete response (CR) was considered when all target lesions disappeared. Partial response (PR) was considered when the sum of diameters of all target lesions was reduced of at least 30%. Progressive disease (PD) was considered when the sum of diameters of all target lesions was increased of at least a 20%, or one or more new lesions appeared. Stable disease (SD) was considered when the sum of diameters of all target lesions reduced < 30%, increased < 20%, or did not change.

Toxicity was assessed with Common Terminology Criteria for Adverse Events version 5.0.

Statistical analysis

Age and survival were reported as median and ranges; frequencies were reported as percentages. Kaplan-Meier non parametric estimates was used for OS and PFS analysis, reporting survival probability on the y axis and time in months on the x axis. Chi square test, Student's *t* test, and log-rank test were used for statistical significance, and $P \leq 0.05$ was used to indicate statistically significant differences.

RESULTS

Sample characteristics

The study included 158 consecutive patients, 58 (37%) of these received CHT + mEHT and 100 (63%) CHT alone. The two sub groups had similar characteristics concerning gender distribution, presence and site of metastases, and previous surgery (Table 1). Some differences were found between CHT + mEHT and CHT groups in median age (64 *vs* 69 years, $P = 0.013$), previous RT (2% *vs* 12%, $P = 0.023$), number of previous CHT lines (2 Lines: 19% *vs* 35%, $P = 0.037$), and type of chemotherapy. Gemox was the most used chemotherapy in CHT + mEHT group ($P = 0.004$), whereas FOLFOX and FOLFIRINOX were used only in CHT group ($P < 0.05$).

Survival

Median OS was greater for CHT + mEHT group than CHT group (19.5 mo *vs* 11.02 mo, $P < 0.001$); also PFS was improved (12 mo *vs* 3 mo, $P < 0.001$) for the CHT + mEHT group (Figure 1A and B).

Tumor response

The association of mEHT resulted also in an improvement of tumor response at the 3 mo time point, with a disease control rate (DCR) 95% *vs* 58% ($P < 0.001$) in CHT + mEHT group and CHT group, respectively (Table 2). CHT + mEHT group, in particular, had a greater PR (52% *vs* 14%, $P < 0.001$) and lower PD (5% *vs* 58%, $P < 0.001$) than CHT group.

Adverse effects and safety

Each patient received an average of 13 (range = 4-28) sessions of mEHT. Out of a total of 754 mEHT delivered sessions, the safety assessment of mEHT showed a limited number of adverse events 23/754 (4%). mEHT toxicity consisted of skin pain in 15 (3%) patients and burns in 8 (1%).

All these side effects were G1-G2 intensity and resolved with local medications and discontinuation of treatment for 1 wk. All patients were evaluated before and after mEHT with electrocardiogram and cardiac ultrasound. No one had cardiac toxicity.

DISCUSSION

Hyperthermia has been used as cancer therapy for decades, especially for its benefits in enhancing chemotherapy and radiotherapy efficacy[6,14-22]. mEHT has been more recently introduced among hyperthermia methods, targeting malignant cell membranes and the extracellular matrix and overcoming the issue of homogenous tissue heating[7,8]. The efficacy of mEHT was shown for several types of tumors, including pancreatic cancer, increasing tumor response and survival[5,9,15-21].

The current study showed that mEHT improved OS (19.5 mo *vs* 11.02 mo, $P < 0.001$) and PFS (12 mo *vs* 3 mo, $P < 0.001$), resulting also in an increased tumor response with DCR 95% *vs* 58% ($P < 0.001$) than CHT alone. Toxicity was comparable in the two study groups and hyperthermia-related adverse events were mainly G1-2.

The beneficial effect of hyperthermia on survival of locally advanced pancreatic cancer was reported if combined with chemotherapy by Tschoep-Lechner, reporting in 23 patients an OS 12.9 mo (95%CI: 9.9-15.9) and a DCR in 16 patients with available CT scans of 50%[20].

Similar results are observed when associated to chemo-radiotherapy (CRT). Three studies, in particular, showed OS of 8.8-15 mo *vs* 4.9-11 mo ($P < 0.05$) in the association group than CRT alone group and PFS = 18.6 mo *vs* 9.6 mo ($P = 0.01$)[17-19]. The association of CHT to RHT also result in encouraging survival: Median OS of 12.9-17.7 mo, 1-year OS = 41% and 2-year OS = 15%[15-17]. Similar survivals are reported by other four studies on mEHT for the treatment of locally advanced pancreatic carcinoma, OS of 8.9-19 mo and PFS of 3.9-12.9 mo[9-11]. The results of the present study were in agreement with the above data, showing OS of 19.5 mo and PFS of 12 mo for CHT + mEHT group. In OS analysis, the survival curve was crossed; this may be due to the fact that stage III-IV pancreatic cancer has always had a poor outcome and, inevitably, when comparing the curves of two treatments, they cross because all patients had died.

Improvements were reported also in tumor response for locally advanced pancreatic carcinoma a consequence of the association of CHT to mEHT with a DCR of

Table 1 Sample characteristics

	CHT + mEHT, *n* (%)	CHT, *n* (%)	*P* value
Median age (range)	64 (38-82)	69 (34-92)	**0.013**
M	39 (67)	57 (57)	0.204
F	19 (33)	43 (43)	
Non-Metastatic	16 (28)	37 (37)	0.223
Metastatic	42 (72)	63 (63)	
Site of Metastases			
Liver	40 (61)	51 (70)	0.164
Peritoneum	16 (24)	6 (8)	
Lymph nodes	9 (14)	6 (8)	
Lung	1 (2)	8 (11)	
Previous surgery	14 (24)	24 (24)	0.981
Previous RT	1 (2)	12 (12)	**0.023**
Previous CHT	52 (90)	52 (52)	**< 0.001**
Number of previous CHT lines			
1	31 (60)	25 (48)	0.334
2	10 (19)	18 (35)	**0.037**
> 3	11 (21)	9 (17)	0.619
Type of CHT			
Gemox	28 (54)	12 (23)	**0.004**
Gemcitabine	12 (23)	7 (13)	0.205
Gemcitabine abraxane	16 (31)	15 (29)	0.830
FOLFIRINOX	0 (0)	5 (10)	**0.022**
FOLFOX	0 (0)	4 (8)	**0.041**
Other	2 (4)	9 (17)	

Significative values were reported in bold. mEHT: Modulated electro-hyperthermia; CHT: Chemotherapy; RT: Radiotherapy.

Table 2 Tumor response at 3 mo

	CHT + mEHT, *n* (%)	CHT, *n* (%)	*P* value
DCR	53 (95)	40 (58)	**< 0.001**
PR	29 (52)	13 (14)	**< 0.001**
SD	24 (43)	27 (28)	0.064
PD	3 (5)	56 (58)	**< 0.001**

Significative values were reported in bold. mEHT: Modulated electro-hyperthermia; CHT: Chemotherapy; DCR: Disease control rate; PR: Partial response; SD: Stable disease; PD: Progressive disease.

95% that were in agreement with previous data: DCR of 71%-96%[8-11] and resulted higher than that reported by CHT associated with local hyperthermia, showing a DCR of 50%-61%[17-19]. The level of hyperthermia related toxicity was 5% as reported by other studies, showing G2 pain and a skin rash and 5% grade III–IV toxicity[15,18]. These data suggest that RHT increases CRT and CHT benefit both in terms of median OS and in DCR in pancreatic cancer with a low toxicity.

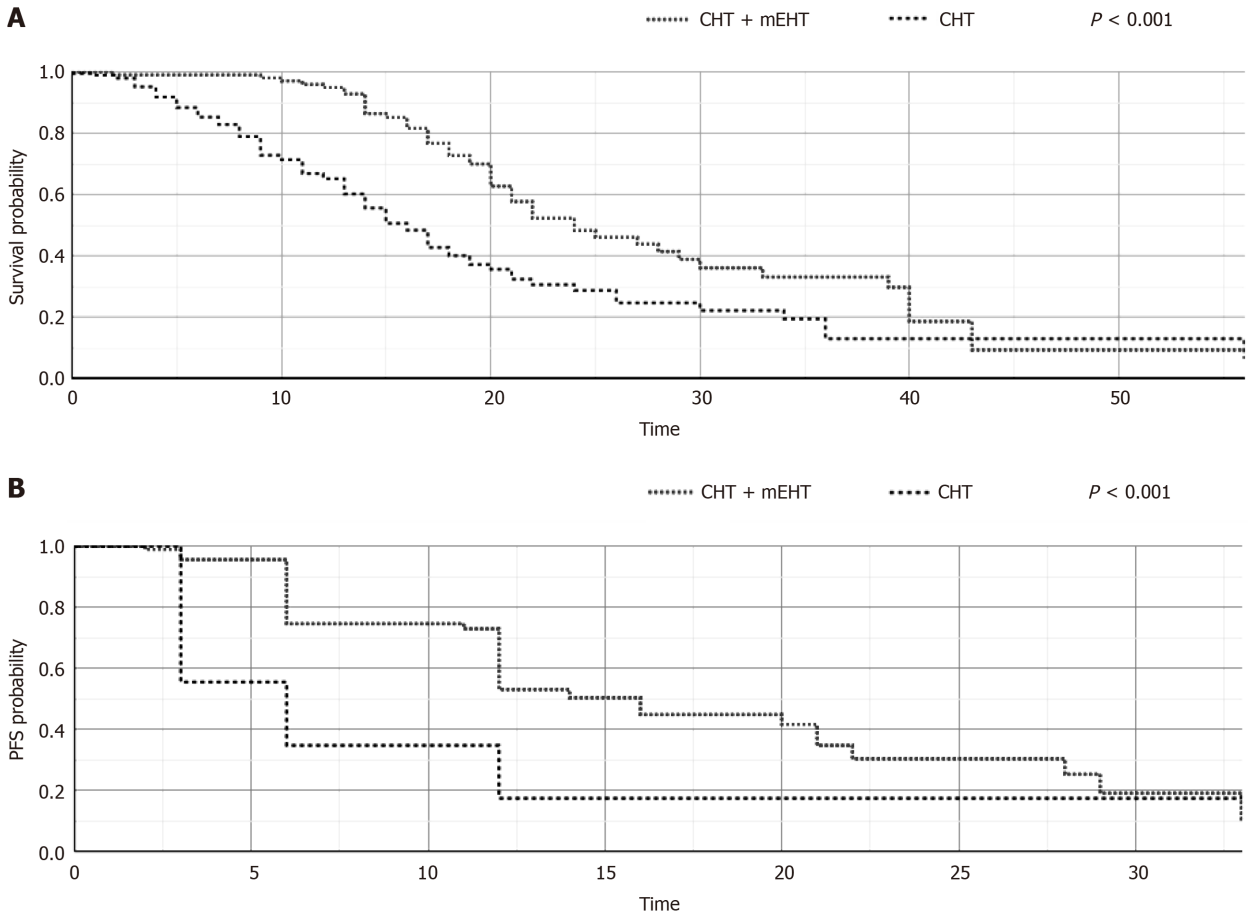


Figure 1 Overall survival (A) and progression free survival (B) analysis of the two study groups. mEHT: Modulated electro-hyperthermia; CHT: Chemotherapy; PFS: Progression-free survival.

The main limitations of this study were due to the observational nature of this study that resulted in some imbalances distribution between the two subgroups as concerning number of patients and median age, since CHT group had a greater number of subjects (100 *vs* 58) that were of median older age (69 years *vs* 64 years) than CHT + mEHT group. Age should not be considered an issue, since the improvement of survival and tumor response was observed also in older patients (> 65 years) with stage III and IV pancreatic cancer[9]. Further prospective, randomized trials on a larger number of patients are required to develop these initial data.

CONCLUSION

The addition of mEHT to systemic CHT improved OS and PFS and local tumor control with comparable toxicity.

ARTICLE HIGHLIGHTS

Research background

Hyperthermia has been used as cancer therapy for decades, especially for its benefits in enhancing chemotherapy and radiotherapy efficacy. Modulated electro-hyperthermia (mEHT) is a relatively new method of hyperthermia that targets malignant cell membranes and the extracellular matrix, overcoming the issue of homogenous tissue heating.

Research motivation

The efficacy of mEHT is known for several types of tumors, including pancreatic cancer. Pancreatic cancer has a poor prognosis, and the combination of mEHT with

chemo- and/or radiotherapy might be important to increase tumor response and improve survival.

Research objectives

The aim of this study was to compare outcomes of chemotherapy (CHT) alone or in association with mEHT for the treatment of locally advanced pancreatic cancer.

Research methods

Data were collected retrospectively from a cohort of 158 consecutive patients with stage III-IV pancreatic cancer that were treated with CHT alone (63%) or in combination with mEHT (37%) from 2003 to 2019. These data included patients' characteristics, type of chemotherapy, previous surgery or radiotherapy, tumor response, survival, progression free survival, and adverse events.

Research results

The evaluation of survival showed that CHT + mEHT group had a longer overall (19.5 mo *vs* 11.02 mo, $P < 0.001$) and progression free (12 mo *vs* 3 mo, $P < 0.001$) survival. The association of mEHT improved also tumor response with disease control rate 95% *vs* 58% ($P < 0.001$). Toxicity was comparable in the two study groups and hyperthermia-related adverse events were mainly G1-2.

Research conclusions

The results obtained in this study provided new evidence that mEHT improved survival and tumor response, delaying the progression insurgence. The introduction of mEHT, moreover, did not influence chemotherapy tolerability, and hyperthermia-related adverse events were limited.

Research perspectives

This observational study provides further evidence that mEHT association to chemotherapy can enhance its benefit in pancreatic cancer patients. Further studies are required to confirm these results in a large cohort study and to evaluate treatment safety and efficacy.

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CASE REPORT

Anaplastic myxopapillary ependymoma: A case report and review of literature

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Author contributions: Kanetsuna Y performed the histological evaluation; Kanno H drafted the manuscript, the table and figures; Shinonaga M evaluated the clinical data and reviewed the manuscript.

Informed consent statement: Written informed consent was obtained from the patient before surgical treatment. In addition, all procedures performed in this study were in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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Abstract

BACKGROUND

Myxopapillary ependymoma (MPE) is a pathological grade I tumor that arises in the filum terminale. MPE with anaplastic features is extremely rare, and only 5 cases have shown malignancy at the time of recurrence.

CASE SUMMARY

The patient (a 46-year-old woman) had undergone a MPE operation 30 years ago. After subtotal resection of the tumor located in L4-S1, it had a solid component that extended to the adjacent subcutaneous region. Histologically, the tumor consisted of a typical MPE with anaplastic features. The anaplastic areas of the tumor showed hypercellularity, a rapid mitotic rate, vascular proliferation, and connective tissue proliferation. Pleomorphic cells and atypical mitotic figures were occasionally observed. The MIB-1 index in this area was 12.3%. The immunohistochemical study showed immunoreactivity for vimentin, glial fibrillary acidic protein and S100. The morphological pattern and immunohistochemical profile were consistent with anaplastic MPE. The patient tolerated surgery well without new neurological deficits. She underwent local irradiation for the residual tumor and rehabilitation.

CONCLUSION

Although extremely rare, anaplastic MPE occurs in both pediatric and adult patients, similar to other ependymomas. At a minimum, close monitoring is recommended, given concerns about aggressive biological potential. In the future, further study is needed to determine the WHO classification criteria and genetic indicators of tumor progression. The possibility of malignant transformation of MPE should be taken into account, and patients with MPE should be treated with care and follow-up.

Peer-review report's scientific quality classification
Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Core Tip: Myxopapillary ependymoma (MPE) is a pathological grade I tumor that develops in the filum terminale. MPEs with anaplastic features are extremely rare; only 5 cases have shown malignancy when they relapsed. Here we report a case of MPE with anaplastic features in local late recurrence in a 46-year-old woman and review anaplastic MPE in the published literature. MPEs have the potential for malignant transformation after a long period of time despite being a pathological grade I tumor. Therefore, the possibility of malignant transformation of the MPE should be considered, and patients with MPE should be treated carefully and monitored over a long period of time.

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INTRODUCTION

Myxopapillary ependymomas (MPEs, grade I) account for 9%-13% of ependymal tumors and about 83% of ependymomas are found in the area of the filum terminale [1]. MPEs are typically encapsulated, slow-growing benign neuroepithelial tumors that occur primarily in regions of the medullary conus and filum terminale, and removal without breaking the capsule is curative[2,3]. The average age at presentation is approximately 36 years, with a significant male prediction[1]. They differ morphologically and biologically from other ependymomas and often require immunohistochemical analysis to distinguish them from phenotypically similar tumors[4]. Despite an overall favorable prognosis and classification as a grade I tumor, MPEs have been associated with distant metastases, subarachnoid disseminations and local late recurrences[5-8].

In the 2016 WHO classification of central nervous system tumors, ependymal tumors are classified into the following five subtypes: MPE and subependymoma, and MPE (grade I), classic ependymoma (grade II), RELA fusion protein positive ependymoma (grade II / III) and anaplastic ependymoma (grade III)[1]. MPEs account for 9%-13% of ependymal tumors and around 83% of ependymomas are found in the area of the filum terminale[2]. MPEs are usually benign, slow growing neuroepithelial tumors that occur predominantly as intradural neoplasms in the region of the medullary conus, cauda equine, and filum terminale, although rare occurrences have been reported in the neck, thoracic spinal cord, lateral ventricles, and cerebral parenchyma[3-6,9]. MPEs are usually encapsulated and removal without damaging the capsule is curative[2,3]. Distant metastases from MEPs in the brain parenchyma and other organs have also been reported[10-20]. They differ morphologically and biologically from other ependymomas and immunohistochemical study is often required for differential diagnosis from chordomas or chondrosarcomas that morphologically resemble ependymomas[6]. The mean age at manifestation is around 36 years, with a male predominance[1]. Anaplastic features in glial tumors including ependymomas include frequent mitotic figures, hypercellularity, necrosis, vascular proliferation, and pleomorphologic cytoplasm and nuclei[21]. However, classification and grading of ependymomas with anaplastic features are historically controversial. Hence, their diagnosis is difficult and subjective. MPEs with anaplastic features are usually locally invasive. They frequently tend to disseminate to other areas of the brain and spinal cord *via* cerebrospinal fluid (CSF), and more frequently recur with a shorter survival[1,21]. Grade II gliomas often transform into more malignant types[22]. However, grade I gliomas rarely transform into more malignant types[23], and MPEs have only shown malignant transformation in 4 cases[24]. MPEs with anaplastic features are extremely rare in the literature (20 cases)[25-29] and recurrent MPEs have

only shown malignant transformation in 5 cases[29]. We report the case of a 46-year-old woman with MPE with anaplastic features and local ultra-late recurrence. Clinical and histopathological findings are described and the malignant transformation of the MPE is discussed.

CASE PRESENTATION

Chief complaints

Lumbar to sacral pain and weak legs for 6 mo.

History of present illness

The patient (a 46-year-old woman) had lumbar to sacral pain and leg weakness for 6 mo and attended the Atami Hospital of the International University of Health and Welfare.

History of past illness

The patient underwent a subtotal resection for a myxopapillary filum ependymoma 30 years ago by the same surgeon.

Personal and family history

No other relevant personal or family history.

Physical examination

The neurological examination showed bilateral muscle weakness of the gastrocnemius, anterior tibia and urethral sphincter as well as sensory disturbances in areas of L4, L5 and S1.

Laboratory examinations

Laboratory examinations showed normal levels of all parameters tested.

Imaging examinations

Magnetic resonance imaging (MRI) (Figure 1) and computed tomography (CT) imaging (Figure 2) studies showed a mass that occupied most of the spinal canal from L2 to S1 and extended into the adjacent subcutaneous region.

FINAL DIAGNOSIS

Examination of the tumor with hematoxylin and eosin staining showed neoplastic cells with round nuclei and clear cytoplasm in the background of the fibrillar stroma. These neoplastic cells formed two transition structures: A typical MPE area and a high quality area with anaplastic features. The low-grade area of the tumor had the following appearance: Typical MPE area with a somewhat poor and pointed arrangement with Alcian blue-positive myxoid matrix. The MIB-1 index in this area was 5.1%. On the other hand, the high-grade anaplastic areas of the tumor showed hypercellularity, a rapid mitotic rate, vascular proliferation, and connective tissue proliferation. Pleomorphic cells and atypical mitotic figures were occasionally seen. The MIB-1 index was 12.3%. The immunohistochemical study showed immunoreactivity for vimentin, glial fibrillary acidic protein (GFAP) and S100, but no immunoreactivity for epithelial membrane antigen and chromogranin A (Figure 3). The morphological pattern and immunohistochemical profile were consistent with an MPE with anaplastic features[29]. The combination of areas of typical MPE-appearing tumor interspersed with areas of ependymoma with anaplastic features was consistent with the diagnosis of MPE with anaplastic features. The final diagnosis was MPE with anaplastic features (Figure 4).

TREATMENT

Prior to surgical treatment, written informed consent was obtained from the patient. After placing the patient in the prone position, a midline incision was made at 15 cm from L2 to S2. After exposing the rostral end of the previous laminectomy at L2, the

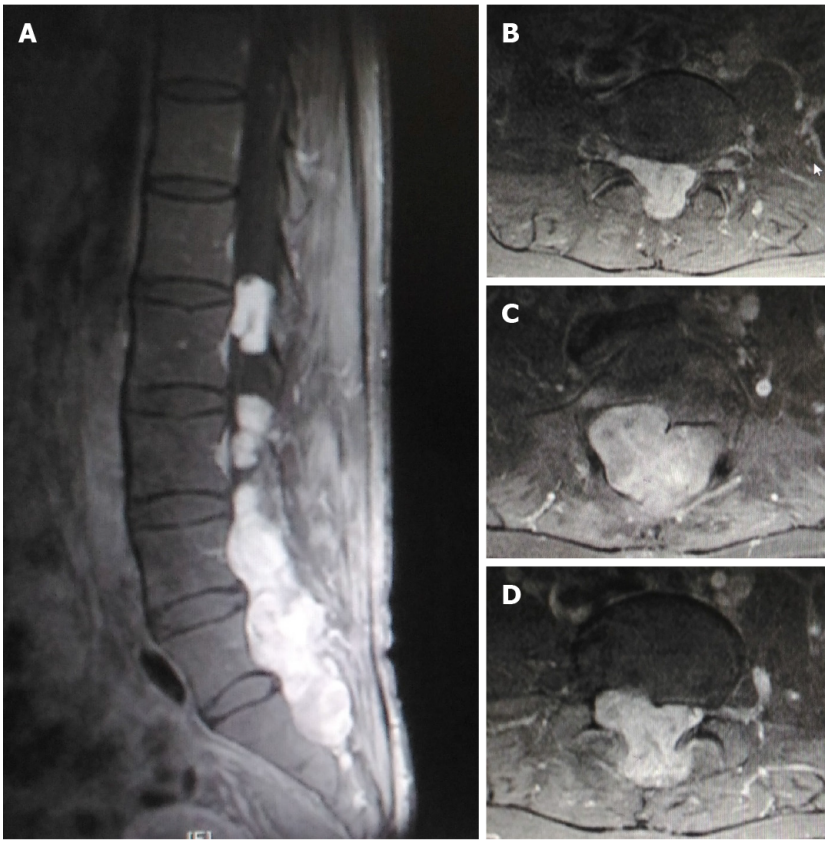


Figure 1 Gadolinium-enhanced T1 weighted magnetic resonance imaging at ultra-late recurrence of a myxopapillary ependymoma. A: Sagittal magnetic resonance imaging (MRI) demonstrated an enhanced mass localized at L3-S1 in the intraspinal canal. A part of the mass extended into the spine axis and dorsal extraspinal tissues; B-D: Axial MRI demonstrated a slightly enhanced mass from intracanal to extraspinal regions.

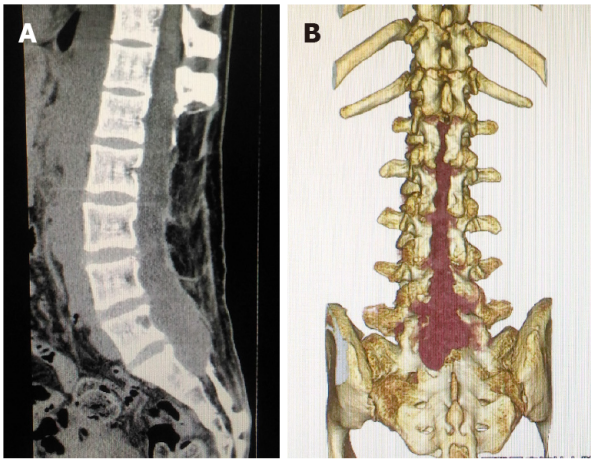


Figure 2 Computed tomography at ultra-late recurrence of a myxopapillary ependymoma. A: A plain sagittal image demonstrated the previous laminectomy area (L1-S1); B: Three-dimensional image demonstrated a recurrent tumor (dark red) in the previous laminectomy area (L1-S1).

previous laminectomy area up to S2 was exposed. In addition, the lamina sacralis was partially drilled. The dura mater was adhered to the granulated soft tissue and opened, but no CSF was found. The encapsulated tumor was observed (Figure 3). The tumor was dark red mixed with gray and appeared to be highly fibrous and hemorrhagic. The filum terminale was compressed by the tumor at the L2 level. Part of the filum penetrated the tumor and was scarified by fibrous restriction due to the difficult dissection. The tumor was debulked with a Cavitron ultrasonic surgical aspirator (Integra Life Science, Dublin, Ireland) and a subtotal resection was performed. Postoperatively, the patient was well without any new neurological deficits.

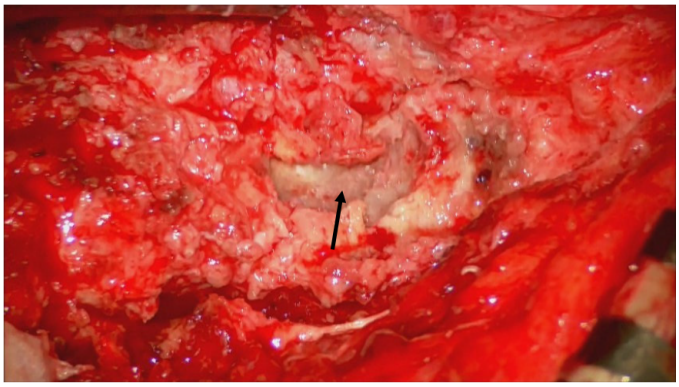


Figure 3 Intraoperative photograph showing an encapsulated tumor (arrow) surrounded by granulated tissues. Right side: Caudal; Left side: Rostral.

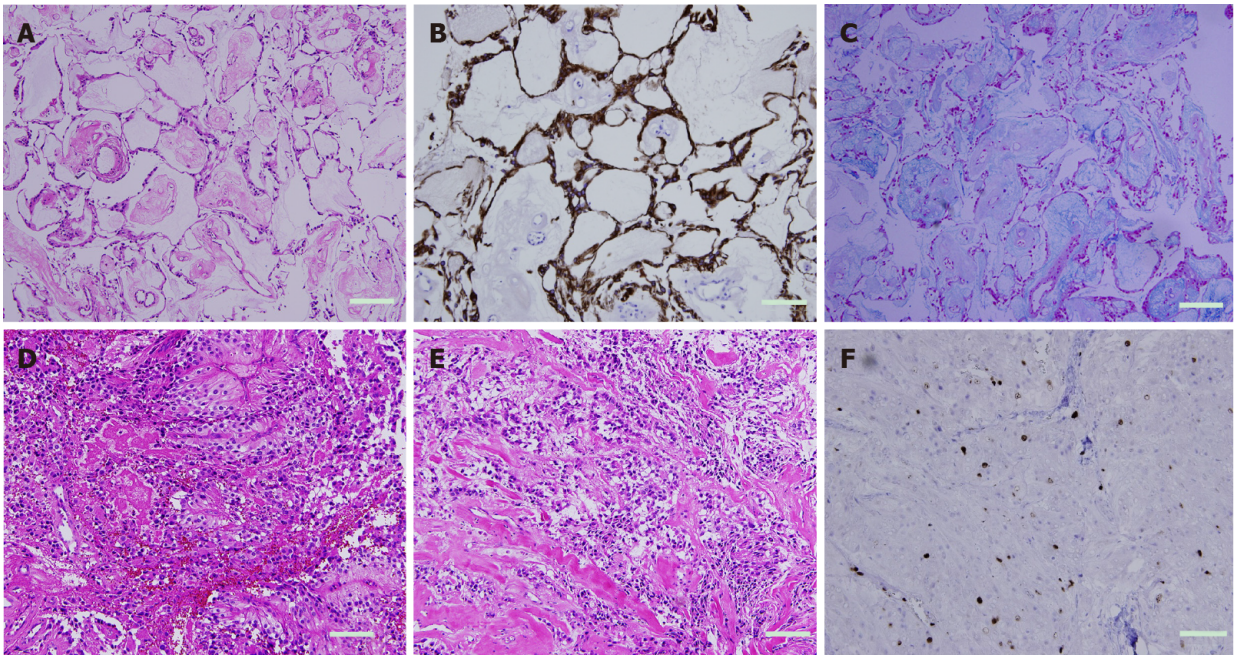


Figure 4 Microscopic appearance of the tumor. Magnification: 200 ×; scale bar: 50 μm. A: Microphotography with hematoxylin and eosin (HE) stain showed the appearance of a typical myxopapillary ependymoma including much mucoid; B: Immunohistochemical microphotography with anti-glial fibrillary acidic protein (GFAP) antibody showed distinct expression of GFAP; C: Microphotography with Alcian blue stain showed much Alcian blue positive mucoid; D and E: Microphotography with HE stain showed anaplastic features such as hypercellularity, rapid mitotic rate, vascular proliferation, and connective tissue proliferation; F: Immunohistochemical microphotography showed a high MIB-1 labeling index (12.3%) in the area with anaplastic features.

OUTCOME AND FOLLOW-UP

Postoperatively, the patient showed no new neurological deficits; and bladder and bowel functions were intact. She presented unchanged motor strength in the lower extremities. She had local radiation of the residual tumor and rehabilitation. She was examined again and showed no deterioration.

DISCUSSION

Pathological and genetic anaplastic MPE

According to a recent report[24], anaplasia of MPE was defined on the basis of histopathological findings that are similar to the criteria currently used to define anaplasia in classical ependymomas, that is, they have at least two of the following features: Mitosis per 10 high power field (HPF), MIB-1 labeling index > 10%, microvascular proliferation and spontaneous necrosis. In the present case, the MIB-1

index of 12.3% corresponds to previous reports. In addition, endothelial proliferation was found in the present case. These pathological findings are compatible with anaplastic MPE. The immunohistochemical description of MPE has been reported, which, in agreement with the present case, consists of a positivity for GFAP and vimentin[16,30,31]. In the present case, interestingly, the histopathological features showed anaplastic features mixed with those of three different components of low-grade ependymoma including MPE. This finding could suggest that an original MPE cell has the potential to become different types of ependymomas that are molecularly different[32]. Recent genetic analyses have shown that MPE is characterized by genome-wide polyploidy, often between several chromosomes[33]. MPE shows specific losses of chr16 and chr12 and increases of chr4, chr9 and chr18, while the classical grade II ependymoma shows a specific loss of chr16 and an increase of chr12 [34]. MPE differs molecularly, transcriptionally and histologically from classical 2nd degree ependymoma. Gene expression profiling also showed that MPEs have a Warburg phenotype[33] and increased gene expression of HOXB13 compared to non-MPEs[34]. The specific familial, epigenetic, or environmental cause that predisposes to malignant transformation of MPE has not been identified. In contrast to grade II gliomas, which have a high progression to high-grade gliomas, grade I gliomas such as pilocytic astrocytoma, ganglioglioma, and MPE rarely undergo malignant transformation with a maximum incidence of 10%[22,23]. Malignant transformation occurred spontaneously or after radiation therapy in these tumors. The specific familial, epigenetic, or environmental cause that predisposes to malignant transformation of the MPE has not been identified.

Clinical features of anaplastic MPE

Similar to previous reports, the present case showed a large mass occupying the spinal canal. In spite of an overall favorable prognosis and classification as a grade I tumor, MPEs have been associated with distant metastases, subarachnoid disseminations, and local late recurrences in almost half of all patients, regardless of adequate resection[3,4,6,7,35-40]. In 20 reported cases of anaplastic MPE, including this case, the age of the patients ranged from 0.9 to 57 years, with a mean of 24.7 years. The majority of patients with anaplastic MPE were under 20 years old, while in a study of 183 patients with classic MPE the mean age at diagnosis was 35.5 ± 15.8 years. CSF dissemination and involvement of adjacent tissue were observed in 50% and 50% of anaplastic MPEs, while distant metastases to the spinal cord and brain were observed in 9.3% and 6% of anaplastic MPEs, respectively[6]. Other studies on classical MPEs have reported higher rates of CSF dissemination of 35% to 57%[7,41], particularly in pediatric cases of MPE and including pediatric dissemination at first presentation in 14%-58% of pediatric cases[8,41]. In the present case, aggressive clinical features such as soft tissue invasion were identified, while distant metastases were not observed. Therefore, many of these patients, particularly those with aggressive clinical features, can undergo radiation therapy. Frequent surveillance scans of patients with MPE are recommended for early detection of recurrent disease with anaplastic features. The 5-year survival rate of patients with non-MPE anaplastic ependymoma has been reported to be less than 20%. The classification of ependymal tumors is currently difficult and according to the current WHO guidelines of questionable clinical benefit[9].

The histopathological criteria for anaplastic ependymoma are as follows: Mitoses (> 4/10 per HPF), hypercellularity, endothelial proliferation, and necrosis[42]. In addition, it has been suggested that anaplastic ependymoma could be defined by the presence of two of these parameters[42]. Large retrospective series of MPE have been conducted, including a large multi-institutional series with 183 patients[6] who had a 10-year overall survival (OS) of 92.4% and a 5- and 10-year progression-free survival (PFS) of 69.5% and 61.2%, respectively. Local MPE relapse occurred in 84% of patients and leptomeningeal spread was observed in 9.3% of patients.

A strong correlation was found between surgical capsule injury and recurrence[43]. A 10-year OS of over 90% was recently confirmed in an epidemiology, surveillance, and outcome analysis of 773 MPE patients[44]. Presacral MPE showed a worse prognosis than MPE in the filum terminale/cauda equina.

Management of anaplastic MPE

According to the 2017 EANO guideline for ependymal tumors, with the exception of gross-total removal (GTR), no factors have been defined that influence the prognosis of spinal cord ependymomas other than MPE[45]. Advances in microsurgical techniques have enabled *en bloc* GTR, which is the standard treatment for spinal cord ependymoma. GTR mostly resulted in good functional outcomes, and a good functional result was related to small tumor size and little neurological deficit at

surgery. Therefore, surgery at an early stage should be considered[46,47]. If GTR cannot be performed due to infiltration of the spinal cord or nerve roots, postoperative local radiation therapy is often used. Multivariate analysis showed that the tumor grade and the extent of resection were independent prognostic factors for OS and PFS, and that radiation therapy extended PFS in patients undergoing subtotal removal (STR). Studies suggest that doses > 50 Gy lead to either better or equivalent results[48,51]. With respect to conventional chemotherapy, it is reported that continuous oral etoposide for recurrent intramedullary ependymomas is well tolerated[53]. Bevacizumab may be of clinical benefit in some patients[52]. Large retrospective series of MPE suggested that the extent of resection was an important independent factor in forecasting local control, while younger age (< 36 years) was a negative prognostic factor. On the other hand, the irregular shape surrounding nerve roots and the formation of a myxoid matrix are related to the risk of postoperative neurological disability[43]. In the treatment of MPE, it has been shown that postoperative radiation therapy with high doses (≥ 50 Gy), compared to patients treated with only one procedure, leads to better local control and longer PFS without significant late toxicity [51,52]. In a small series of adult patients with spinal MPE, it was shown that patients treated with GTR followed by adjuvant radiation therapy had better local control than patients treated with GTR alone[3]. Although patients often have a disseminated tumor and/or develop recurrent or progressive disease after treatment[54], OS is estimated to be 97% and 95% after 5 and 10 years, respectively[55]. A recent series from the Johns Hopkins Hospital[56] showed a significant reduction in local failure in patients receiving radiation therapy after STR or GTR. A smaller series[57] also confirmed good local control with surgery and radiation therapy compared to GTR alone. In summary, according to the 2017 EANO guideline for ependymal tumors[45], the following key recommendations for the treatment of WHO grade I and anaplastic (WHO grades II and III) MPEs are proposed: Total resection is the goal of MPE-surgery (II B). MRI after surgery should be performed to assess the extent of the resection (N/A). Since all patients with newly diagnosed ependymoma are at risk of CSF dissemination, disease staging, including craniospinal MRI and CSF cytology, is recommended after surgery (N/A). A watch-and-wait strategy is recommended for WHO grade II ependymomas after total resection (IIIC). In the case of anaplastic (WHO grade II and III) MPE, postoperative radiation therapy with doses of 45-54 Gy is recommended, irrespective of the extent of the resection (IIIC). Following the incomplete resection of an MPE of WHO grade I, postoperative local radiation therapy with a dose of 50 Gy is recommended (IIB). In the event of relapse, reoperation, re-radiation, and chemotherapy should be considered (IIIC). As the risk of later relapse exists, patients should be followed up with an enhanced MRI over a long period (N/A).

Review of anaplastic MPE

Anaplastic MPE is an extremely rare event and 19 cases have been reported in the literature[24-29]. In 20 reported cases of anaplastic MPE, including this case, the patients ranged in age from 0.9 years to 57 years, with a mean of 24.7 years. The majority of patients with anaplastic MPE were under 20 years old, while in a study of 183 patients with classic MPE the mean age at diagnosis was 35.5 ± 15.8 years. Of the previously reported MPEs with anaplastic features, anaplasia was present in only 5 cases with only one recurrence. In addition, the present case is the most recent relapse in the literature. Similar to previous reports, the present case showed a large mass occupying the spinal canal, and aggressive clinical features such as soft tissue invasion were identified, while distant metastases and CSF dissemination were not observed (Table 1). Despite being histological grade 1 tumors, typical MPEs are often associated with distant metastases, CSF disseminations and local late recurrences. Therefore, patients with a typical MPE can be irradiated after surgery, and it is recommended that patients with MPE be monitored regularly to detect distant metastases or local recurrences[1,2]. In the present case, the patient was not irradiated after the previous operation 30 years ago due to the lack of histological anaplasia. If the patient had been irradiated after the previous operation, recurrence could have been avoided. A 5-year survival rate of less than 20% has been reported for anaplastic ependymoma without MPE. The histopathological parameters of anaplastic ependymoma include > 4 mitoses per 10 HPF, endothelial proliferation, hypercellularity, and necrosis, and the presence of two of these parameters define the diagnosis of anaplastic ependymoma[42]. However, the classification of ependymomas with pathologically anaplastic features remains controversial. Hence, diagnosing these tumors is difficult and subjective at times. Anaplastic ependymomas often recur and survival time is reduced[2]. In addition, the tumors often spread to other regions of the central nervous system

Table 1 Summary of the clinicopathologic features of myxopapillary ependymomas with anaplasia

Ref.	Age (yr) at MPE with anaplasia (age at typical MPE)	Sex	Location of MPE with anaplasia	Adjacent tissues involved	CSF diss	MIB-1 Index	MVP	Treatment	
								Initial	Recurrence
Awaya *et al*[25], 2003	15	M	Th12-L2	No	No	10%	Yes	GTR	No
Beschorner *et al* [26], 2007	3	M	Subcutaneous sacrococcyx	Yes	No	40%	Yes	GTR	No
Vega-Orozco *et al* [27], 2011	38 (22)	M	Inguinal node metastasis	Yes	Yes	NA	Yes	STR, RT	RT
Chakraborti *et al* [28], 2012	0.9	F	Subcutaneous sacrococcyx	Yes	No	70%	Yes	GTR, CT	No
Huynh *et al*[29], 2018	24	F	L2-3	Yes	Yes	8%-38%	Yes	GTR	GTR
Lee *et al*[24], 2019	6	F	L4-S1	No	No	20%	Yes	GTR, RT	No
Lee *et al*[24], 2019	7	F	T12-L3	No	No	11%	Yes	STR, RT	No
Lee *et al*[24], 2019	10	M	L1-2	Yes	No	34%	Yes	GTR, RT	No
Lee *et al*[24], 2019	10	M	S1-2	No	No	15%	Yes	GTR, RT	No
Lee *et al*[24], 2019	11	M	L4-S3	No	No	14%	Yes	GTR	No
Lee *et al*[24], 2019	12	M	Lumbo-sacral	Yes	Yes	10%	Yes	GTR	GTR, RT
Lee *et al*[24], 2019	13	M	L1-2	No	Yes	8%	Yes	STR, RT	No
Lee *et al*[24], 2019	20 (16)	F	L3-S1	No	Yes	10%	No	GTR	RT
Lee *et al*[24], 2019	32 (31)	M	S1	No	Yes	10%	Yes	STR, CT, RT	RT
Lee *et al*[24], 2019	40	F	4th ventricle	No	No	20%	Yes	STR, RT	No
Lee *et al*[24], 2019	45 (31)	F	Extraspinal pelvic	Yes	Yes	40%	Yes	STR, RT	GTR, CT
Lee *et al*[24], 2019	50	M	L5-S3	Yes	Yes	10%	Yes	GTR, RT	No
Lee *et al*[24], 2019	55	F	L1-2	No	Yes	20%	Yes	GTR, RT	No
Lee *et al*[24], 2019	57 (45)	M	T8-L5	Yes	Yes	26%	Yes	STR, RT	GTR, RT
Kanno *et al*, 2021	46 (16)	F	L4-S1	Yes	No	12%	Yes	STR	STR, RT

CSF diss: Cerebrospinal fluid dissemination; MVP: Microvascular proliferation; F: Female; M: Male; GTR: Gross total resection; STR: Subtotal resection; Chem: Chemotherapy; RT: Radiation therapy; CT: Chemotherapy; NA: Not available.

through CSF and are usually locally invasive. Recently, it was suggested that the diagnostic criteria for anaplastic MPE should be based on histopathological findings that include at least two of the following parameters: > 5 mitoses per 10 HPF, > 10% MIB-1 labeling index, spontaneous necrosis, and, microvascular proliferation. In the present case, the MIB-1 index of 12.3% is compatible with previous reports. In addition, endothelial proliferation was found in the present case. These pathological findings were consistent with anaplastic MPE[32]. Malignant transformation of MPE can occur in both pediatric and adult patients and is associated with either relapse, local invasion, CSF spread, or metastatic disease. These anaplastic clinical features indicate a more aggressive biological potential than classic MPE. Therefore, regular close observation is recommended. Further studies are needed to refine the proposed assessment criteria for anaplastic MPE and to identify the genetic biomarkers of tumorigenesis and malignant transformation of MPE[33].

CONCLUSION

MPE has the potential for malignant transformation after a long period of time despite

being a pathological grade I tumor. Therefore, the possibility of malignant transformation of MPE should be considered, and patients with MPE should be carefully managed and followed up over a long period of time.

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CASE REPORT

Foreign body granulomas mimic peritoneal dissemination caused by incarcerated femoral hernia perforation: A case report

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Abstract

BACKGROUND

Foreign body granuloma (FBG) is a well-known type of granulomatous formation, and intraabdominal FBG (IFBG) is primarily caused by surgical residues. Multifocal IFBGs caused by gastrointestinal perforation is an extremely rare and interesting clinicopathological condition that resembles peritoneal dissemination. Here, we present a case of IFBGs mimicking peritoneal dissemination caused by bowel perforation and describe the value of intraoperative pathological examinations for rapid IFBG diagnosis.

CASE SUMMARY

An 86-year-old woman with an incarcerated femoral hernia was admitted to the hospital and underwent operation. During the operation, the incarcerated ileum was perforated during repair due to hemorrhage necrosis, and a small volume of enteric fluid leaked from the perforation. The incarcerated ileum was resected, and the femoral hernia was repaired without mesh. Four months later, a second operation was performed for an umbilical incisional hernia. During the second operation, multiple small, white nodules were observed throughout the abdominal cavity, resembling peritoneal dissemination. The results of peritoneal washing cytology in Douglas' pouch and the examination of frozen nodule sections were compatible with IFBG diagnosis, and incisional hernia repair was performed.

CONCLUSION

IFBGs can mimic malignancy. Intraoperative pathological examinations and operation history are valuable for the rapid diagnosis to avoid excessive

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Core Tip: Multifocal intraabdominal foreign body granulomas (IFBGs) caused by gastrointestinal perforation are clinically rare and mimic peritoneal dissemination. An 86-year-old woman underwent an operation to treat an incarcerated femoral hernia; however, the incarcerated ileum was perforated due to hemorrhage necrosis, resulting in incarcerated ileum resection. After 4 mo, a second laparoscopic operation was conducted for an umbilical incisional hernia; however, small, white nodules were identified throughout the entire abdominal cavity, mimicking peritoneal dissemination. Using intraoperative cytology and frozen sections, the nodules were diagnosed as IFBGs. IFBGs sometimes mimic peritoneal dissemination, and intraoperative pathological examinations are effective for rapid diagnosis.

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INTRODUCTION

A foreign body granuloma (FBG) is a tissue reaction caused by retained foreign bodies, and intraabdominal FBGs (IFBGs) are typically caused by materials used in a previous operation, such as surgical sutures, gauze, and sponges[1-3]. However, in rare cases, bowel perforation, bile or gallstone spillage, and foreign bodies unrelated to operations, such as fish bones, can result in IFBGs[4-6]. Furthermore, IFBGs sometimes occur multifocally and can closely resemble peritoneal dissemination, leading to misdiagnosis[5,7]. Here, we present a case of IFBGs mimicking peritoneal dissemination 4 mo after an operation to treat an incarcerated and perforated femoral hernia.

CASE PRESENTATION

Chief complaints

An 86-year-old woman presented to our hospital with anorexia and vomiting for 3 d.

History of present illness

Her medical history was unremarkable.

History of past illness

Past appendectomy.

Personal and family history

No special in personal and family history.

Physical examination

Severe pain with redness and a palpable bulge was observed in the right inguinal area.

Laboratory examinations

The laboratory workup showed a white blood cell count of 17.1 × 10^9/L and C-reactive protein levels at 6.13 mg/dL.

Imaging examinations

Abdominal computed tomography (CT) showed a right incarcerated femoral hernia with small bowel obstruction.

FINAL DIAGNOSIS

FBG (see TREATMENT section).

TREATMENT

The laparoscopic repair of an incarcerated femoral hernia was attempted; however, the incarcerated ileum was perforated during the repair because it had become necrotic. The necrotic ileum was resected, and the right femoral hernia was repaired using the McVay procedure (Figure 1). Although postoperative intensive care for septic shock and disseminated intravascular coagulation was necessary, the patient was discharged one month after the operation. The patient revisited our hospital 3 mo later due to an umbilical incisional hernia, and laparoscopic incisional hernia repair was performed. During the operation, the hernia orifice, 11 cm in diameter, was identified at the umbilical wound, and multiple small white nodules expanded throughout the entire abdominal cavity (Figure 2). We suspected the peritoneal dissemination of some tumors. Because no observable tumor could be located in the abdominal cavity, peritoneal wash cytology of Douglas's pouch was performed, and frozen sections of the nodules were examined. The cytology was negative for malignancy (Figure 3), and the frozen sections showed a multinucleated giant cell containing a foreign body, surrounded by inflammatory cells, fibrosis, and granulomatous formations (Figure 4), which was compatible with an FBG reaction. Therefore, laparoscopic intraperitoneal onlay mesh repair with hernia orifice closure (IPOM-plus) was performed.

OUTCOME AND FOLLOW-UP

The postoperative clinical course was uneventful, and no malignant findings were observed on chest-to-abdominal enhanced CT or gastrointestinal and colon endoscopy. No malignancy was detected, and no recurrence of hernia was observed during 7 mo of follow-up.

DISCUSSION

FBG is a chronic immune reaction induced by the presence of a foreign body. IFBG is typically caused by retained surgical residues, bowel perforation, bile spillage, gallstones, or fish bones[1,2,4-6,8]. Suture granulomas are the most common type associated with surgical residuals, although gauze and sponges have also been reported[1-3]. IFBGs occasionally resemble tumorous nodules and sometimes occur multifocally, which can mimic peritoneal dissemination[2,8]. IFBG is also well-known to be caused by glove powder used in operations. IFBGs associated with lycopodium, talcum, and starch powders from surgical gloves have previously been reported, and these IFBGs can be quite difficult to differentiate from cancerous nodules[7,9,10]. Akita *et al*[5] reported that food starch released from gastrointestinal perforations could cause multiple IFBGs, mimicking peritoneal dissemination; they reported a case in which tenderness and guarding over the entire abdomen was observed due to a gastric cancer perforation, which was treated surgically. Two months later, many small, white granulomas mimicking peritoneal dissemination were observed, particularly in the upper abdominal cavity, which were diagnosed as IFBGs based on frozen sections during the second operation. In the pathological findings, saburra was observed at the center of the granuloma.

In the present case, multiple small, white nodules that expanded throughout the entire abdominal cavity were observed during the second operation, which were extremely difficult to differentiate from peritoneal dissemination. A multinucleated giant cell containing a foreign body similar to saburra and surrounded by inflammatory cells and granuloma formation was observed pathologically; thus, these

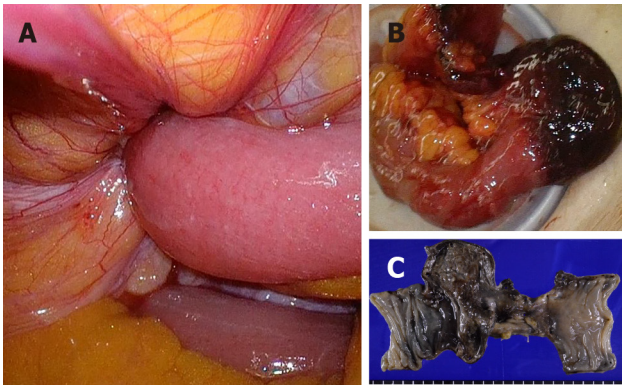


Figure 1 The necrotic ileum was resected, and the right femoral hernia was repaired using the McVay procedure. A: The ileum was incarcerated into the right femoral hernia; B: The incarcerated ileum became necrotic; C: The resected ileum showed hemorrhage necrosis over a 10 cm length.

nodules were diagnosed as IFBGs and were thought to be caused by food starch, similar to the previously reported case[5], particularly as no evidence of malignancy was identified either during or after the operations.

FBG is one type of non-infectious granulomatous reaction, characterized by the presence of a foreign body surrounded by granuloma formations in foreign body giant cells. FBGs have also been associated with granuloma formation due to an excessive immune response to foreign bodies, potentially related to allergic reactions and microbiological factors[11]. Intriguingly, in the present case, the IFBG expanded to the entire abdominal cavity at the time of the second operation, although the amount of enteric fluid that leaked from the perforation was limited to a small range and was localized within the lower abdominal cavity during the first operation. Because the lower abdominal cavity was rinsed after the resection of the incarcerated ileum during the first operation, the remaining food particles released from the small enteric fluid leak should have been minimized and at low concentrations, which suggested that each IFBG was either caused by an extremely minute food starch quantity or represented an allergic response, resulting in the accumulation of microbiological factors. Furthermore, the characteristic histological findings of FBG were very supportive of an IFBG diagnosis, especially during the operation. The misdiagnosis of IFBGs as malignancies can potentially lead to ineffective or unnecessary treatments. A previous case reported IFBGs mimicking peritoneal dissemination 3 mo after laparoscopic cholecystectomy, resulting in the wedge resection of the liver and transverse colon and omentectomy, and IFBGs were later diagnosed pathologically as likely due to bile or gallstone spillage[6]. As reported by Akita *et al*[5], intraoperative cytology and frozen sections are valuable for the rapid diagnosis of IFBGs. In the current case, these quick pathological examinations performed during the operation provided an important and powerful method for differentiating IFBGs from peritoneal dissemination.

The physiologic activation of histiocytes generally occurs within 24-48 h[12]; however, the time required for granulomatous formation resembling nodules remains unclear. In the current case, 4 mo was sufficient for the observed granulomatous formation. The percentage of granulomas observed during operations decreases gradually, from 37% if the previous operation was performed within 6 mo to 18% when the previous operation occurred more than 2 years prior[1]. Granulomas that resemble peritoneal dissemination can form within 2 mo, according to Akita *et al*[5], and this occurred within 4 mo in the current patient. Therefore, the interval between first and second operations also supports the diagnosis of IFBG.

CONCLUSION

In conclusion, multiple IFBGs caused by bowel perforations are associated with a very rare clinicopathological condition and can mimic peritoneal dissemination. A history of past operations and the interval between the current and past operations can be helpful for distinguishing between IFBGs and peritoneal dissemination, and intraoperative cytology and frozen sections are extremely valuable methods for the rapid diagnosis of IFBG, which can prevent misdiagnosis and avoid the use of ineffective or excessive treatments.

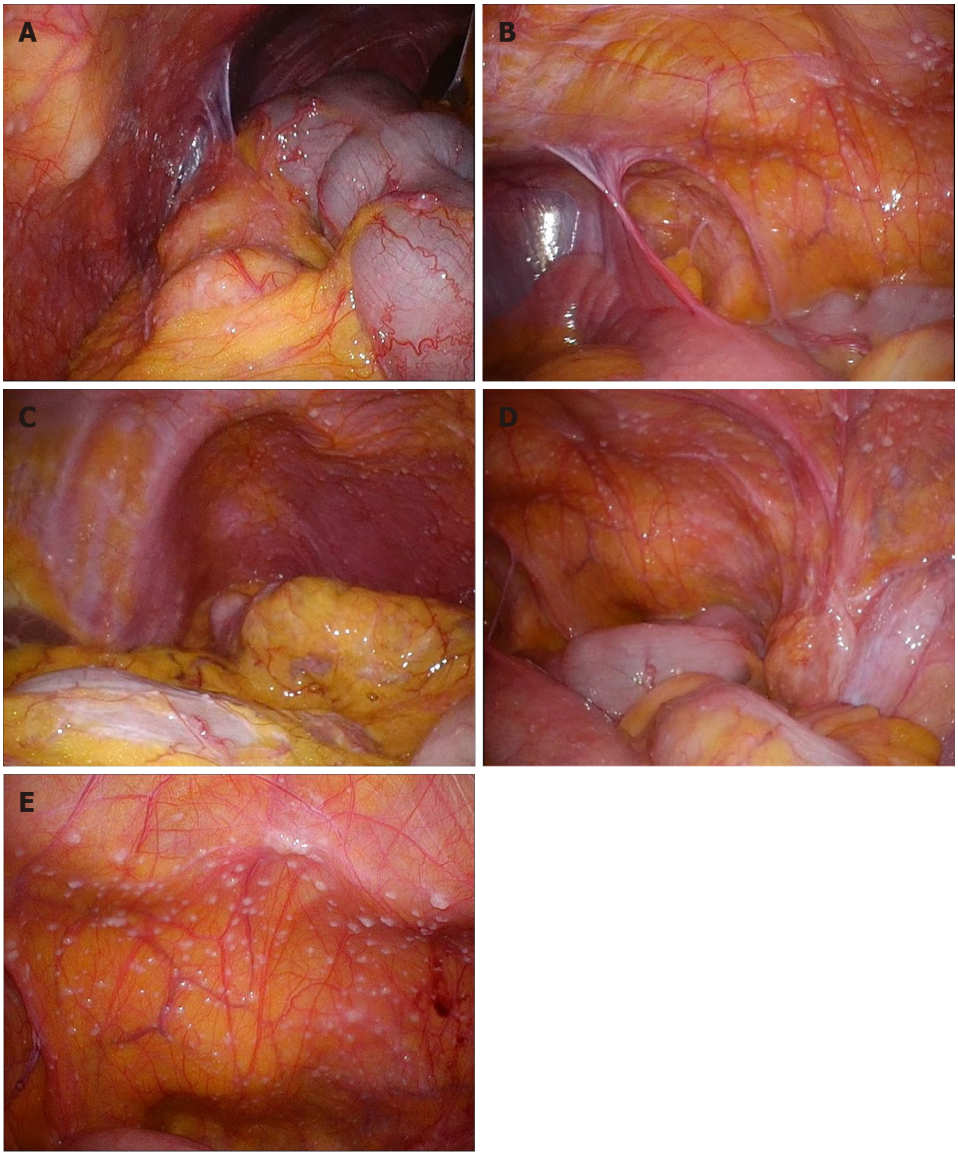


Figure 2 Multiple small white nodules mimicking peritoneal dissemination were observed throughout the entire abdominal cavity during the second operation. A: Right upper abdominal cavity; B: Right lower abdominal cavity; C: Left upper abdominal cavity; D: Left lower abdominal cavity; E: Back side of the bladder.

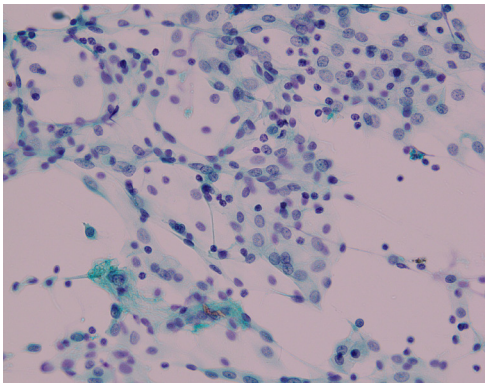


Figure 3 Papanicolaou stain of the peritoneal washing cytology of Douglas' pouch showed only inflammatory cells (× 40).

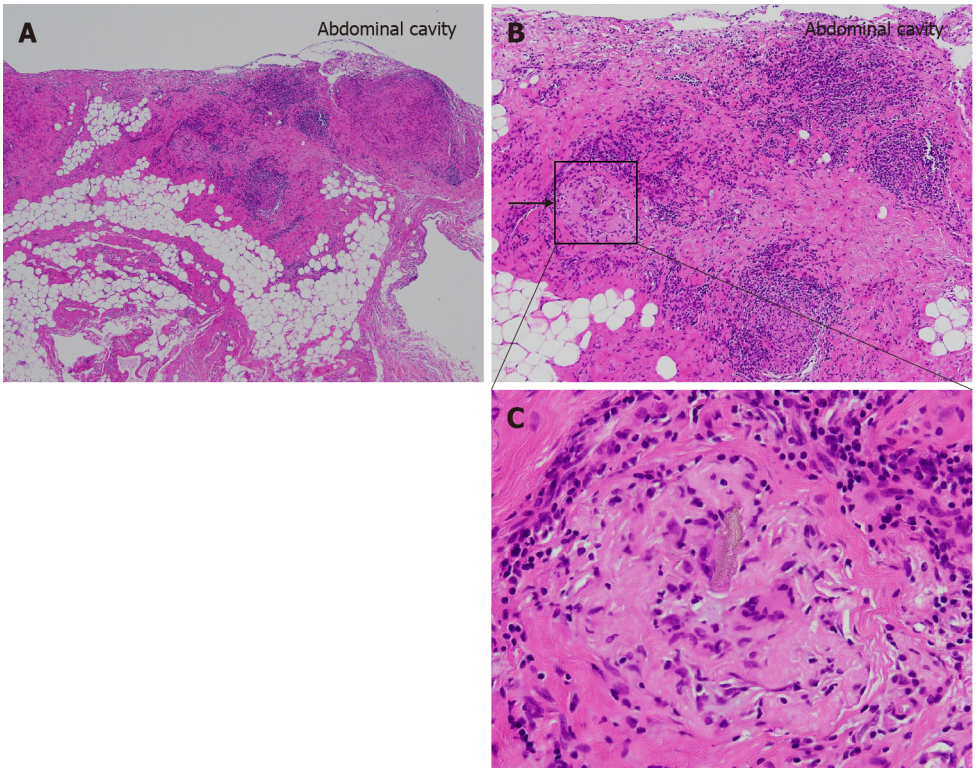


Figure 4 Pathological findings of the small white nodule. Hematoxylin and eosin stain of the nodule. A multinucleated giant cell with a foreign body (arrow) surrounded by monocytes, lymphocytes, and granuloma formations. A: × 40; B: × 100; C: × 200.

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OPINION REVIEW

Advances and controversies in the management of early stage non-small cell lung cancer

Angel Cilleruelo-Ramos, Esther Cladellas-Gutiérrez, Carolina de la Pinta, Laura Quintana-Cortés, Paloma Sosa-Fajardo, Felipe Couñago, Xabier Mielgo-Rubio, Juan Carlos Trujillo-Reyes

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Abstract

Complete resection continues to be the gold standard for the treatment of early-stage lung cancer. The landmark Lung Cancer Study Group trial in 1995 estab-

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lished lobectomy as the minimum intervention necessary for the management of early-stage non-small cell lung cancer, as it was associated with lower recurrence and metastasis rates than sublobar resection and lower postoperative morbidity and mortality than pneumonectomy. There is a growing tendency to perform sublobar resection in selected cases, as, depending on factors such as tumor size, histologic subtype, lymph node involvement, and resection margins, it can produce similar oncological results to lobectomy. Alternative treatments such as stereotactic body radiotherapy and radiofrequency ablation can also produce good outcomes in inoperable patients or patients who refuse surgery.

Key Words: Video-assisted thoracoscopic surgery; Sublobar resection; Radiofrequency ablation; Stereotactic radiosurgery; Early stage; Lung cancer

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Core Tip: Complete resection continues to be the gold standard for the treatment of early-stage lung cancer. Lobectomy remains the gold standard for the treatment of early-stage non-small cell lung cancer, but there is a growing tendency to perform sublobar resection in selected cases. Alternative treatments such as stereotactic body radiotherapy and radiofrequency ablation can also produce good outcomes in inoperable patients or patients who refuse surgery.

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INTRODUCTION

Complete resection continues to be the gold standard for the treatment of early-stage lung cancer. The landmark Lung Cancer Study Group trial in 1995 established lobectomy as the minimum intervention necessary for the management of early-stage non-small cell lung cancer (NSCLC), as it was associated with lower recurrence and metastasis rates than sublobar resection and lower postoperative morbidity and mortality than pneumonectomy[1]. The development of lung-sparing techniques (*e.g.*, sleeve resection with vascular and/or bronchial reconstruction) has reduced the number of pneumonectomies performed and with this the risk of adverse outcomes, as the proportion of pneumonectomies is a quality indicator in thoracic surgery[2].

While lobectomy remains the gold standard for the treatment of early-stage NSCLC, there is a growing tendency to perform sublobar resection in selected cases, as, depending on factors such as tumor size, histologic subtype, lymph node involvement, and resection margins, it can produce similar oncological results to lobectomy[3]. Two randomized clinical trials comparing lobectomy and sublobar resection are currently underway: The United States Cancer and Leukemia Group B trial (CALGB 140503)[4] and the Japanese JCOG0802/WJOG4607L trial[5]. The results so far have shown no significant differences in postoperative morbidity or mortality, but as discussed in greater detail below, data on survival and pulmonary function are pending.

The use of minimally invasive techniques for the surgical treatment of early-stage NSCLC has increased in recent years. Video-assisted thoracoscopic surgery (VATS) is the current procedure of choice for most resections in this setting. A recent nationwide cohort study conducted in Spain reported that over 50% of recent anatomic lung resections had been performed by VATS[6]. The main advantages of VATS compared with open surgery are decreased postoperative pain, fewer postoperative complications, and in some cases even, better oncological outcomes. There are, however, substantial geographic variations in the use of VATS.

Advances in VATS techniques and the design of specific surgical material have led to a progressive reduction in the number of incisions required. Most thoracic surgeons now use between one and three incisions and describe similar oncological results[6,7]. Subxiphoid VATS is another minimally invasive technique associated with good outcomes when performed by teams with extensive experience in VATS; it has been linked to a lower incidence of postoperative neuropathic pain[8].

The increasing adoption of VATS has favored its use in more locoregionally advanced lung cancers. Data from large series of angio-bronchoplastic or extended lung resections performed by experienced thoracic surgeons show similar outcomes to thoracotomy[9].

Good outcomes have also been described with robotic-assisted thoracoscopic surgery in the setting of anatomic resections, although the cost-effectiveness of the technique is not so clear[10].

As we discuss below, alternative treatments such as stereotactic body radiotherapy (SBRT) and radiofrequency ablation (RFA) can also produce good outcomes in inoperable patients or patients who refuse surgery.

ROLE OF SUBLOBAR RESECTION IN LUNG CANCER

Anatomic sublobar resections have produced comparable oncological results to lobectomy in the treatment of tumors < 2 cm without nodal involvement or distant metastasis[11]. These favorable results have led to an increased use of segmentectomy, which, depending on tumor stage and resection margins, can produce similar oncological results to lobectomy in selected patients[12].

Anatomic segmentectomy is oncologically more valuable than atypical (wedge) resection in early-stage cancer as it permits the performance of hilar and mediastinal lymph node dissection[13].

Its main advantage, however, is its parenchyma-sparing effect, which results in better postoperative respiratory function than lobectomy. In view of the above, anatomic sublobar resection can be considered an appropriate treatment for patients with compromised respiratory function unable to tolerate standard lobectomy. Patients considered to be at high operative risk include patients with FEV_1 < 50% or DLCO < 50% and elderly patients with impaired lung function, pulmonary hypertension, and poor left ventricular function[14,15].

Compared with lobectomy, VATS sublobar resection has been linked to shorter hospital stays and drainage times, a lower incidence of supraventricular arrhythmia, and fewer postoperative respiratory complications[11].

In certain cases, anatomic segmentectomy involves a higher risk of air leakage when electrocautery is used for intersegmental plane dissection (as reported by several Japanese groups)[5,13]. Air leakage is not common when absorbable sutures are used, which is the case in most lung resections.

The only randomized prospective trial to compare lobectomy and sublobar resection in T1N0M0 lung cancer (the Lung Cancer Study Group trial) concluded that patients treated with sublobar resection had a higher risk of locoregional recurrence and death[1]. It should be noted, however, that these results were published in 1995 and that lung tumors are now diagnosed earlier.

Several retrospective studies published since 2000 have reported good oncological outcomes in patients with small peripheral tumors (stage I and < 2 cm) treated with segmentectomy[13,16-19].

As mentioned, the ongoing CALGB[4] and Japanese[5] trials have not detected any differences between lobectomy and sublobar resection for postoperative morbidity or mortality, but survival and pulmonary function outcomes are not yet available[4,13,20].

Thus, it remains to be determined whether segmentectomy is a valid alternative to lobectomy for the treatment of early-stage NSCLC in patients fit for both procedures[4,5].

POSTRESECTION ADJUVANT THERAPY IN NSCLC

Thirty percent of lung cancer patients have early-stage disease when diagnosed. The standard treatment is surgery, followed or not by chemotherapy with or without radiotherapy.

Data from retrospective series show that less invasive surgical procedures result in fewer complications, allowing earlier initiation of chemotherapy, but do not appear to have an impact on overall survival (OS).

Postoperative radiotherapy in stage I and II NSCLC is indicated for patients with positive margins. According to the recent results of the phase III LUNG ART trial, postoperative radiotherapy did not have any beneficial effects in patients with pathologic mediastinal involvement (N2), in addition, it induced high levels of toxicity. Chemotherapy, however, was associated with a 5.4% increase in OS at 5 years, regardless of age [hazard ratio (HR) = 0.89]. Chemotherapy is indicated for resected stage II and IIIA NSCLC[21], but its use in stage I disease is more controversial. The standard treatment is four cycles of doublet cisplatin-based chemotherapy. The only clinical trial to investigate the use of carboplatin in this setting reported negative results[22]. Survival outcomes, however, are poor, mainly because of high rates of distant recurrence. Five-year OS rates range from 73% for stage IB disease to 65% for stage IIA disease, 56% for stage IIB disease, and 41% for stage IIIA disease[23]. It is therefore important to continue to explore new treatments and prognostic and predictive biomarkers.

Attempts to improve treatment outcomes with the addition of antiangiogenics[24] or vaccine-based therapy[25] have been unsuccessful. The potential benefits of immunotherapy are being investigated, as good results have been reported for adjuvant immunotherapy in more advanced stages of disease and other types of tumor [26]. Ongoing trials include PEARLS (pembrolizumab), BR31 (durvalumab), ANVIL (nivolumab), Impower 010 (atezolizumab), and Canopy-A (canakinumab). No results, however, are available yet. Immunotherapy, both alone and combined with chemotherapy, has shown promising results in the neoadjuvant setting. Chemoimmunotherapy has significantly improved complete and major pathological responses in NSCLC (by approximately 36% and 65%, respectively) and has also led to downstaging in over 70% of patients[26,27]. It remains to be determined whether immunotherapy is more effective as a neoadjuvant or adjuvant treatment[28].

Agents targeting driver mutations are being investigated in multiple trials, but results are still pending. We do have results from the ADAURA trial, where patients with completely resected *EGFR* mutation–positive NSCLC, regardless of whether or not they had received prior chemotherapy, were randomized to receive osimertinib [a third-generation tyrosine-kinase inhibitor (TKI)] or placebo for 3 years. The progression-free survival (PFS) outcomes for patients with stage II and IIIA disease in the osimertinib group were unprecedented, with an HR for disease recurrence or death of 0.17. In addition, the benefits were observed in all the subgroup analyses. The adverse events were to be expected based on the experience with this drug. Osimertinib was also associated with a reduction in brain recurrences (HR = 0.18)[29]. These results were sufficient for the United States Food and Drug Administration to approve osimertinib as an adjuvant treatment for NSCLC with *EGFR* mutations. Recent results from another trial showed that icotinib, a first-generation TKI, improved PFS (HR = 0.36) in patients with resected stages II and IIA disease; results on OS have not been published yet[30]. Nonetheless, in the CTONG trial of adjuvant treatment with gefitinib, the improvement observed for PFS was not carried over to OS, reflecting previous findings for other targeted therapies. It remains to be seen whether osimertinib will achieve a survival benefit in the ADAURA trial.

Little has been reported on the use of biomarkers in this setting, as they were not a requirement in most of the trials conducted to date. Thus, the potential values of *BRCA1* and of *ERCC1* and thymidylate synthase were not validated in the respective SCAT and ITACA trials. Contradictory results have been reported for the prognostic value of PDL-1 expression and tumor mutational burden[31-33]. Nonetheless, next-generation sequencing is a promising strategy for the detection of residual disease after surgery[34,35]. A recent meta-analysis showed that residual molecular disease detected by circulating tumor DNA analysis after complete resection was associated with a higher risk of recurrence and death.

Despite the available evidence, treatment should always be individualized, with careful assessment of risks and benefits, particularly in the current scenario of COVID-19[36].

SBRT IN EARLY-STAGE LUNG CANCER

SBRT is a high-precision technique that delivers high doses of radiation over a short period of time[37]. Conceptually derived from cranial stereotactic radiosurgery, it is

now used in multiple anatomic locations. It is the treatment of choice for early-stage lung cancer in medically inoperable patients or patients who refuse surgery, with a 5-year local control rate of 90%[38]. It improves survival in older patients and reduces the number of untreated patients. When SBRT is not feasible, hypofractionated radiotherapy is preferred to conventionally fractionated schedules[39]. Acute toxicity is rare in SBRT, and includes mild fatigue 1-2 wk after treatment; quality of life is rarely affected[40]. The risk of severe toxicity is low[41], and the most common adverse effect is decreased lung capacity. SBRT can be highly toxic in patients with a history of interstitial lung disease and its use should be assessed by a multidisciplinary committee. Late adverse effects include pain, rib fractures, dyspnea, and ventricular tachycardia[38]. Other effects are esophagitis, epithelitis, and brachial plexopathy. Complications are largely influenced by tumor location and size, radiation dose and target volume[42]. Pathological confirmation is not always possible, and some authors have suggested that up to 16% of lung nodules may be benign[4].

SBRT has certain technical characteristics that need to be taken into account when planning and administering treatment. Four-dimensional computed tomography (CT) is recommended for preoperative simulation, and multiple beams or arcs should be used for planning purposes as they help limit toxicity[43].

Dose schedules for peripheral tumors vary, but mostly consist of 3-8 fractions of 7.5-20 Gy each; results for a dose of 54 Gy in 3 fractions include a 3-year local control rate of 91%, a 3-year disseminated failure rate of 22%[44], a 5-year local control rate of 80%, and a 5-year local control rate of 31%[41]. A phase II trial comparing 30 Gy in 1 fraction and 60 Gy in 3 fractions showed 2-year survival rates of 71% and 61%, respectively, with no differences in toxicity[45]. On comparing 34 Gy in 1 fraction and 48 Gy in 4 fractions, Nagata *et al*[46] found OS rates of 61% and 78%, respectively, and no differences in survival, primary tumor control, or toxicity. In their meta-analysis of 34 observational studies involving 2597 patients, Zhang *et al*[47] determined that the most beneficial dose regimens were those that achieved a biologically equivalent dose of 83.3-146 Gy[47].

Centrally located tumors are tumors located within 2 cm, in any direction, of a critical mediastinal structure, such as the bronchial tree, esophagus, heart, brachial plexus, major vessels, spinal cord, phrenic nerve, and recurrent laryngeal nerve. SBRT is not suitable for ultracentral tumors, but hypofractionated schedules consisting of 6-15 fractions could be considered[48]. Risk-adapted schedules have achieved high local control rates and limited toxicity. Evidence to date shows a 5-year OS rate of 50% and a local control rate of 93%[49,50]. A systematic review of SBRT efficacy and toxicity in centrally located NSCLC showed similar local control and survival rates to those achieved in peripheral tumors.

Three randomized clinical trials have compared SBRT and surgery, although they had problems with accrual. A pooled analysis of the STARS and ROSEL trials showed comparable 3-year recurrence-free survival. Results from the ACOSOG Z4099 trial have not been reported. In the RTOG 0813 trial, 100 medically inoperable patients with central tumors were treated with 50-60 Gy in 5 fractions on alternating days. This resulted in 2-year local control, OS, and PFS rates of 88%, 70%, and 53%, respectively; 15 patients experienced grade 3 or higher toxicity (grade 3, 10 patients; grade 4, one patient; and grade 5, four patients). The standard treatment for patients with operable tumors is surgery, lobectomy, and mediastinal lymph node dissection. The RTOG 0236 [41] and 0915[47] trials showed a 3-year OS rate of 56% over a median follow-up of 4 years and a 5-year OS rate of 40%. The local control and 3-year survival rates were 87.3% and 59.9%, respectively. High recurrence rates, however, were observed in the SBRT group during follow-up[51,52]. Results from the VALOR, SABRTooth, RTOG 3502, and STABLE-MATES trials are pending (Table 1).

When used to treat multiple synchronous tumors *vs* solitary tumors, SBRT offers similar local control and toxicity rates and worse survival rates[53]. The role of SBRT is being investigated in T3-4N0M0 tumors with schedules of 8-10 Gy per fraction in 8-10 fractions. Two-year local control rates of 68%-73.2% have been described[54-56].

A recent study demonstrated that SBRT after contralateral pneumonectomy was safe. Arifin *et al*[57] analyzed 59 studies with a mean follow-up of 25.4 mo and found a mean 1-year OS rate of 80.6%, a 2-year local control rate of 89.4%, and a grade ≥ 3 rate of 13.2%.

RFA IN EARLY-STAGE NSCLC

RFA is a minimally invasive CT-guided procedure originally approved for use in liver

Table 1 Studies analyzing surgery and stereotactic body radiotherapy in non-small cell lung cancer

Ref.	Type	Surgery-RT, No.	Surgery-RT, Local failure	Surgery-RT, PFS	Surgery-RT, OS	Surgery-RT, Toxicity	LoE
Grills *et al* [74], 2010	R	69 wedge resection; 58 SBRT; Unfit for lobectomy	20%-4% (P = 0.07)	65% *vs* 77% (P = 0.37)	87% *vs* 72% (P = 0.01)	Readmission 10%; Pneumonitis 2%; Fracture 11%	3
Varlotto *et al* [75], 2013	R	48 sublobar resection +132 lobectomy; 137 SBRT	At 5 yr 18.8% lobectomy *vs* SBRT 12.2% (P = 0.382); Resection 7.1%	No differences (P = 0.378)	At 5 yr lobectomy *vs* SBRT 33.7%; Resection 86.3% (P = 0.04, P = 0.003)		3
Verstegen *et al*[76], 2013	R	64 VATS; 64 SBRT; 54% inoperable	At 3 yr 3.1% *vs* 1.6% (P = 0.04)	79.7% *vs* 75%	76.9% *vs* 90.8% (P = 0.83)	23.4% *vs* 6.3% G ≥ 3 (P = 0.03)	3
Matsuo *et al* [77], 2014	R	53 sublobar resection; 53 SBRT	At 5 yr 14.1% *vs* 28.3% (P = 0.059)		55.6% *vs* 40.4% (P = 0.124)		3
Zheng *et al* [78], 2014	MA	11921; 7071 surgery; 4850 SBRT	At 1 yr 93% lobectomy *vs* 91.5% sublobar resection *vs* 96.3% SBRT. At 3 yr 85% *vs* 78.4% *vs* 87.8%. At 5 yr 80% *vs* 63.4% *vs* 83.9% (P = 0.45)	At 1 yr 93.5% lobectomy *vs* 90.3% sublobar resection *vs* 87.1% SBRT. At 3 yr 82.9% *vs* 82.1% *vs* 65.8%. At 5 yr 74.8% *vs* 71.2% *vs* 65.8% (P = 0.46)	At 1 yr 92.5% lobectomy *vs* 93.2% sublobar resection *vs* 83.4% SBRT. At 3 yr 77.9% *vs* 80.7% *vs* 56.6%. At 5 yr 66.1% *vs* 71.7% *vs* 41.2% HR = 0.52, 95%CI: 0.2-1.36 for lobectomy and HR = 0.49, 95%CI: 0.19-1.3 for sublobar resection		1
Yu *et al*[79], 2015	R	1078; 711 surgery; 367 SBRT			At 2 yr 77.7% *vs* 59.9% (P = 0.01)	Acute 54.9% *vs* 7.9% (P < 0.001). Chronic 73.9% *vs* 69.7% P = 0.31)	3
Rosen *et al* [80], 2016	R	1781 lobectomy; 1781 SBRT			At 5 yr 59% *vs* 29%; 58% *vs* 40% for patients who refused surgery (P = 0.010)		3
Ma *et al*[81], 2016 (adjusted for operable patients)	MA	6969; 3436 VATS; 4433 SBRT		No differences (P = 0.378)	No differences HR = 2.02, 95%CI: 0.45-3.07 (P = 0.36)		2
Deng *et al* [82], 2017	MA	13598	No differences (P = 0.453)		At 3 yr 68.1% *vs* 47.7% (P < 0.001)		1
Grills *et al* [74], 2010	P. III	222 Lobectomy; 254 SBRT	At 5 yr 5% *vs* 8% (P = 0.388)	At 5 yr 72% *vs* 53% (P = 0.018)	At 5 yr 78% *vs* 61% (P = 0.006)		1
Ackerson *et al* [83], 2018	R	151 surgery; 70 SBRT	At 3 yr 10% *vs* 15% (P = 0.71)	42% *vs* 29% (P = 0.004)	At 3 yr 63% *vs* 35% (P < 0.001)	23%-17%	3
Tamura *et al* [84], 2019	R	141 surgery; 106 SBRT	Higher for SBRT (P = 0.0082)	At 5 yr 69.7%-50.2% (P = 0.036)	At 5 yr 69.7% *vs* 50.2% (P = 0.036)	8.6% surgery; SBRT G ≥ 2 7.5%	3

G: Grade; LoE: Level of evidence; MA: Meta-analysis; P: Phase; OS: Overall survival; RT: Radiotherapy; VATS: Video-assisted thoracoscopic surgery.

tumors. It is a percutaneous technique that consists of applying an alternating current (420-500 kHz) to the tumor tissue, resulting in high temperatures (> 70 °C) that cause tissue necrosis and protein denaturation[58].

Because air is a poor conductor of electricity and a good thermal insulator, the lung is theoretically an ideal site for the application of RFA as the surrounding parenchyma is barely affected[59]. The use of RFA to treat lung tumors was first described by Dupuy *et al*[60] in 2000.

The main advantages of RFA over surgery are that it is minimally invasive (percutaneous technique performed with local anesthesia), can be administered on an outpatient basis or under 24-h hospitalization, and does not require thoracotomy[59].

The use of RFA is limited to the treatment of lesions < 3 cm located in the outer two-thirds of the lung parenchyma. Tumor size affects the homogeneity of the temperature distribution within the lesion. Tumors > 3 cm require the use of several overlapping electrical fields to achieve a high enough temperature, and this increases the risk of complications. As with surgery, a margin of healthy parenchymal tissue must be included in the radiofrequency field, but this is difficult to achieve because of the

thermal insulation effect mentioned above[61-63].

Central lesions carry a higher risk of complications due to their proximity to the bronchial tree, esophagus, and heart. RFA may be less effective when applied to tumors located close to blood vessels with a diameter > 0.3 cm due to what is known as a "heat sink" effect (a cooling effect caused by the constant renewal of blood within the vessel)[59].

The main adverse effects associated with RFA are pneumothorax [the most common complication (11%-67%) following removal of the electrode from the parenchyma], pleural effusion (related to the increase in pleural temperature), hemoptysis, and more rarely, infections, bronchial fistula, and nerve or cardiac injuries.

In a recent meta-analysis comparing RFA and sublobar resection, Chen *et al*[59] analyzed four retrospective studies involving 309 patients: 155 treated with RFA and 154 with sublobar resection. The patients who underwent sublobar resection had significantly higher 1- and 3-year OS and PFS rates (97% *vs* 91% for 1-year OS, 67% *vs* 52% for 3-year OS, 91% *vs* 81% for 1-year PFS, and 67% *vs* 48% for 3-year PFS). Patients in the RFA group had more complications, but they were milder than those seen in the sublobar resection group.

In their prospective phase II trial of 42 patients with inoperable early-stage lung cancer, Palussière *et al*[64] concluded that RFA was a well-tolerated technique with 1- and 3-year local control rates of 84.38% and 81.25%, respectively, and comparable OS rates to those achieved with SBRT. Good tolerability has also been described by other authors[65], including Li *et al*[61] in their meta-analysis of 1989 patients.

Few studies have compared local treatments (RFA and SBRT), and the little evidence that exists is based on unbalanced, retrospective data. Randomized prospective studies are needed. Authors who have compared RFA and SBRT, however, agree that SBRT should be the technique of choice for inoperable early-stage cancer because of its favorable safety profile and greater survival benefits. RFA, in turn, should be reserved for small tumors not located near vessels or mediastinal structures[66-68].

At the molecular level, hypoxia-inducible factor-1α has been proposed as an independent prognostic marker in the setting of RFA, as high levels have been linked to an increased risk of mortality[69].

In conclusion, RFA may be useful for treating inoperable early-stage lung cancer, in particular tumors < 3 cm located far from the mediastinum and vessels with a diameter > 0.3 cm[70,71]. The poorer outcomes reported for RFA compared with sublobar resection may be due to the lack of randomized, prospective studies comparing the two treatments, as studies to date have included patients who are unfit for surgery, that is older, more frail patients with more comorbidities and as a result a worse prognosis[72,73].

CONCLUSION

Complete resection continues to be the gold standard for the treatment of early-stage lung cancer. Lobectomy remains the gold standard for the treatment of early-stage NSCLC, but there is a growing tendency to perform sublobar resection in selected cases. Alternative treatments such as SBRT and RFA can also produce good outcomes in inoperable patients or patients who refuse surgery.

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REVIEW

Liver regeneration biology: Implications for liver tumour therapies

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Abstract

The liver has remarkable regenerative potential, with the capacity to regenerate after 75% hepatectomy in humans and up to 90% hepatectomy in some rodent models, enabling it to meet the challenge of diverse injury types, including physical trauma, infection, inflammatory processes, direct toxicity, and immunological insults. Current understanding of liver regeneration is based largely on animal research, historically in large animals, and more recently in rodents and zebrafish, which provide powerful genetic manipulation experimental tools. Whilst immensely valuable, these models have limitations in extrapolation to the human situation. In vitro models have evolved from 2-dimensional culture to complex 3 dimensional organoids, but also have shortcomings in replicating the complex hepatic micro-anatomical and physiological milieu. The process of liver regeneration is only partially understood and characterized by layers of complexity. Liver regeneration is triggered and controlled by a multitude of mitogens acting in autocrine, paracrine, and endocrine ways, with much redundancy and cross-talk between biochemical pathways. The regenerative response is variable, involving both hypertrophy and true proliferative hyperplasia, which is itself variable, including both cellular phenotypic fidelity and cellular trans-differentiation, according to the type of injury. Complex interactions occur between parenchymal and non-parenchymal cells, and regeneration is affected by the status of the liver parenchyma, with differences between healthy and diseased liver. Finally, the process of termination of liver regeneration is even less well understood than its triggers. The complexity of liver regeneration biology combined with limited understanding has restricted specific clinical interventions to enhance liver regeneration. Moreover, manipulating the fundamental biochemical pathways involved would require cautious assessment, for fear of unintended consequences. Nevertheless, current knowledge provides guiding principles for strategies to optimise liver regeneration potential.

Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Core Tip: The liver has remarkable regenerative potential, allowing recovery from 90% hepatectomy in some rodent models. Current understanding of liver regeneration comes from *in vitro* and animal models. Liver regeneration is controlled by mitogens acting in autocrine, paracrine, and endocrine ways. Complex cross talk occurs between parenchymal and non-parenchymal cells. Regeneration involves hypertrophy and hyperplasia, with both cellular phenotypic fidelity and transdifferentiation, which come into play according to the nature and magnitude of the injury, and the presence of underlying liver disease. Current knowledge provides guiding principles for strategies to optimise liver regeneration potential in the treatment of liver tumours.

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INTRODUCTION

The process of liver regeneration is highly complex, and incompletely understood. Moreover, the components of this complexity are multiple. Firstly, liver regeneration may be triggered by a wide range of diverse injury types, occurring in isolation or combination, and including physical trauma, infection, inflammatory processes, direct toxicity, and immunological insults. Commensurate with the range of injuries, the biochemical mechanisms which trigger liver regeneration in the first place are also diverse, but only partly identified. Second, the response to injury is not only dependent on the type of injury, but also its magnitude. For example, liver growth after 30% partial hepatectomy in the rat model is predominantly by hepatocyte hypertrophy (liver growth by hepatocyte volume increase), in contrast to the hyperplasia (liver growth by hepatocyte proliferation) seen after 70% hepatectomy. The mechanism underlying this observation is poorly understood. Third, even in the context of liver regeneration by proliferation, different pathways are activated depending on the magnitude of the injury and the status of the background liver. Thus, when the default pathway of phenotypic fidelity (hepatocytes dividing to produce more hepatocytes, cholangiocytes dividing to produce more cholangiocytes, and so on) fails, alternative pathways are recruited whereby intrahepatic bipotential cells transdifferentiate to hepatocytes or cholangiocytes to meet the deficit. Fourth, the triggers and drivers to liver regeneration are an expanding multitude of cytokines, hormones, and growth factors (collectively referred to as hepatic mitogens), from hepatic and extra-hepatic sources, acting either synchronously or metachronously, each subject to complicated and ill-defined control mechanisms and feedback loops. The mitogen maelstrom is characterized by much redundancy (ablation of particular mitogens is compensated by others) and overlapping 'biochemical promiscuity' (with mitogens impacting on more than one receptor, or intracellular signalling pathway). This degree of overlap and redundancy is understandably a highly valuable evolutionary adaptation to meet the diverse insults the liver is exposed to. Whilst many mitogens have been identified and characterized, the complexity of the interactions make it extremely difficult to assign quantitative relative contributions or importance. Fifth, the complexity of interactions in mitogenic stimuli is further enhanced by the interplay between parenchymal cells (hepatocytes and cholangiocytes) and non-parenchymal cells [Kupffer cells (KC), hepatic stellate cells (HSC), liver sinusoidal endothelial cells (LSEC)], with the latter group, though present in much smaller numbers, playing critical roles. Sixth, the regenerative response is significantly affected not only by the nature of the injury and its magnitude, but also by the health of the underlying liver. Thus, liver regeneration in the face of established steatosis, steatohepatitis, fibrosis, cirrhosis, or biliary outflow obstruction is much altered to that in healthy liver tissue. Seventh, although the processes driving liver

regeneration are only partially understood, those controlling the stop signals, once the liver has grown sufficiently, are even less well defined. Lastly, although many *in vitro* and animal models are available for the study of liver regeneration, all have their limitations, and their results cannot necessarily be extrapolated to the human situation where information is most limited.

This review provides an overview of liver regeneration biology, and the implications of our current understanding for the treatment of liver tumours. We discuss the subject in separate sections listed below. It is emphasized that the presentation of the subject in this way, though designed to orientate the reader, is somewhat artificial in the context of a biological process characterized by multiple synchronous and overlapping events. There is therefore a degree of overlap between sections, with references made to key events in one section subsequently expanded upon in later ones.

Section 1 describes the models of liver regeneration and provides an account of the in vitro, animal, and human models that provide our current knowledge of liver regeneration.

Section 2 describes the very early events post liver injury (provided by the hepatectomy model) and provides an account of the known early triggers to liver regeneration.

Section 3 provides an account of the multiple hepatic mitogens which contribute to initiating and maintaining liver regeneration.

Section 4 describes the contribution of non-parenchymal cells to liver regeneration.

Section 5 describes the 'alternative pathways' of liver regeneration, in which stem cell trans differentiation is recruited as a mechanism to deal with situations when phenotypic fidelity fails.

Section 6 describes the influence of underlying liver disease to liver regeneration.

Section 7 describes current knowledge of the mechanisms underlying ceasing of liver regeneration.

Section 8 considers how our current knowledge of liver regeneration affects therapy for liver tumours currently and in terms of future developments.

SECTION 1: MODELS OF LIVER REGENERATION

Although the clinician's perspective may aim to use understanding of liver regeneration to optimize and develop therapeutic interventions in humans, much of our current knowledge of liver regeneration is based on animal and *in vitro* models. This section describes the historical evolution of liver regeneration research, the current predominant animal models: Rat, mouse, and zebrafish, the modern tissue culture *in vitro* models, and finally human studies of liver regeneration.

Historical evolution of liver regeneration research

Early research and the flow theory: The history and evolution of animal models used for the study of liver regeneration is described in detail within the excellent review by Mortensen *et al*[1]. The very first liver regeneration research is attributed to Nicolas Eck, a 29-year-old Russian military surgeon, in his investigation of portocaval fistula in dogs[2].

From this early period and into the early 1900s, the prevailing view, referred to as 'the flow theory' hypothesized that liver homeostasis and regeneration could be maintained provided that the liver sinusoids were supplied with mechanical flow of blood, irrespective of its source.

The theory was seemingly supported by experiments showing liver regeneration in dogs after 70% hepatectomy who had undergone total portocaval transposition (thus delivering exclusively systemic venous blood to the sinusoids)[3], and by similar experiments showing liver regeneration in dogs after 42% hepatectomy who had undergone portocaval shunt and arterialization of the hepatic portal stump[4] (thus delivering exclusively arterial blood to the sinusoids). With hindsight, the interpretation of these results was incorrect, in that in both cases, liver regeneration was in fact supported by growth factors of portal origin spilling into the systemic circulation.

The humoral theory: The concept that constituents of portal blood were essential to liver homeostasis and regeneration only gradually gained acceptance, despite early evidence form Hahn who described liver failure in dogs undergoing portocaval shunts [5]. In the 1920s, Rous and Larimore reported that unilateral portal ligation produced ipsilateral atrophy with contralateral hypertrophy in a rabbit model[6]. From the 1960s

onwards many more investigators pursued the idea of portal flow as critical in liver regeneration, in experiments including those of Marchioro *et al*[7], who carried out canine split portocaval transposition in which one portal branch is supplied with venous blood from the inferior vena cava and the second portal branch receives portal blood, showing atrophy and hypertrophy of the respective parts of liver parenchyma. Furthermore it was demonstrated that adjusting flow and oxygenation alone did not, in a dog model, compensate for the absence of portal blood[8].

Characterising portal blood constituents: With the recognition of the importance of portal blood came an impetus to define the source and nature of vital portal blood constituents. Thus splanchnic portal flow separation experiments were carried out separating portal flow of distal stomach, duodenum, pancreas and spleen from that of small intestine, with the overall finding that the grafts supplied with small intestinal portal flow atrophied, in contrast to those supplied with portal blood from the upper intestinal tract[9,10].

Thereafter, searches for candidate hepato-trophic factors were carried out by infusing individual growth factors and hormones in portal deprived parenchyma to see if rescue could be achieved. In this way, it was demonstrated that insulin infusion into one portal branch of liver after portocaval shunt could partially rescued atrophy of the liver[11], though insulin was unable to prevent liver atrophy following complete splanchnic evisceration[12].

This portocaval shunt rescue model of experimentation allowed the identification of other factors which promoted liver regeneration including thyroxine (T 3), insulin-like growth factor II, transforming growth factor alpha (TGFα) and hepatocyte growth factor (HGF)[13]. Although portal in origin, the systemic blood dissemination of the factors involved in liver regeneration were shown in canine experiments with auto-transplantation of small liver grafts to the jejunal mesentery, then randomising animals to sham surgery or 70% hepatectomy. In contrast to sham surgery, autografts in hepatectomised animals did not atrophy, indicating a growth stimulus *via* the systemic circulation[14].

Similar results were obtained in parabiosis experiments. Thus, using rats with surgically conjoined systemic circulations, partial hepatectomy in one rat, resulted in liver hypertrophy in the non-hepatectomised rat[15,16].

Thus, the early experiments establishing the underlying principles of liver regeneration were performed using predominantly large animal models. In the more recent era, small animal models have preferentially been used because, as well as providing similar physiology and anatomy to the large animal models, they presented advantages in terms of cost, animal husbandry, rapidity of experimentation, and, in the mouse in particular, greater opportunity for genetic modification as an investigative tool. The sections below follow on to describe the rat and mouse models, with the subsequent evolution to the zebrafish model.

It should be emphasised that drawing conclusions from these different models presents additional complexity per se, in that the observations of one species model may not necessarily be extrapolated to the others. Moreover, even within one species, different liver injury types may present differing characteristics. For example, in the mouse model, epidermal growth factor receptor (EGFR) blockade markedly inhibits liver regeneration after paracetamol injury[17], but only delays it after partial hepatectomy (PH)[18].

Current predominant animal models: rat, mouse, and zebrafish

Rat model: The rat model has gained favour over larger animal models (*i.e.*, dogs, rabbits, baboons and pigs) due to advantages in terms of ethics, costs, and practicalities such as husbandry, handling, and shorter experimental times[19] although their size renders surgery more intricate.

As early as 1931, Higgins & Anderson described a standardized technique for partial hepatectomy in rats, which resulted in liver regeneration[20]. Two decades later, Bucher *et al*[15] reported on parabiotic experiments, whereby rats that underwent partial hepatectomy were joined to partner rats with intact livers by way of an abdominal wall anastomosis. The authors found that mitosis increased both within the operated and the intact livers, thus concluding that liver regeneration is influenced by factors in the systemic circulation. In a contemporaneous report of parabiotic rats, Wenneker & Sussman[16] found that liver weight and number of hepatic cells increased both in hepatectomized and "normal" rats, thus reaching the same conclusion. Moolten & Bucher[21] investigated this further by establishing carotid-to-jugular cross-circulation from partial hepatectomy to normal rats, and demonstrating

that DNA synthesis increased in the normal livers, dependent on the extent of hepatectomy in the parabiotic partner. Since these early experiments, a variety of surgical and hepatotoxic rat models have been developed for the study of regeneration in acute and chronic liver disease.

The rat liver consists of four main lobes: middle (38% liver mass), left lateral (30%), right (22%), and caudate (8%)[19]. In descriptions where the paracaval portion is considered separate from the caudate, this amounts to 2% of liver mass. These lobes, and their subdivisions, are analogous to the human liver segments described by Couinaud[22]. Specifically, the caudate lobe (which consists of the Spiegel lobe and paracaval portion) corresponds to the human segments (Sg) I and IX, the left lobe to Sg II, the left component of the middle lobe to Sg III, the right component to Sg IV, V, and VIII, and the right lobe to Sg VI and VII[23].

The classical surgical model involves a 70% (2/3) hepatectomy, as described by Higgins & Anderson[20], and remains the most common surgical model for liver regeneration. Impressively, rat liver can completely regenerate within 8 d of 70% hepatectomy[24]. Variations to this model can result in 5%-97% partial hepatectomies, depending on the combination of liver lobes resected[19]. Impressively, 90% hepatectomy in rats is survivable[25]. Furthermore, survival can (perhaps counterintuitively) be enhanced by suppressing the abrupt early regenerative response of the remnant liver *via* the mitogenactivated protein kinase pathway, thus rendering regeneration linear in the acute phase[26] or by selective bowel decontamination with gentamicin[27]. These phenomena point towards a substantial regenerative reserve in rats, which unfortunately is not found in humans and which limits extrapolation from rodent models to humans. Bile duct ligation (BDL) is another commonly used surgical model, which involves dividing the common bile duct between ligatures, thus providing a model for the study of cholestatic disease[28]. Yet another surgical model is portal branch ligation, after which ipsilateral atrophy and contralateral hyperplasia is observed in rats[29] analogous to human clinical scenarios such as portal vein embolisation (PVE) or associating liver partition and portal vein ligation for staged hepatectomy (ALPPS).

Hepatotoxic models have been extensively studied in both rats and mice, shown in Table 1, with the aim of replicating acute or chronic liver disease. Their mechanisms are also described below in the context of mouse models. The hepatotoxic approach has been used to demonstrate the protective effects of flavonoids[30], thiamine[31] protocatechuic acid[32], *Lactococcus lactis* in probiotic preparation[33] and 5-methoxytryptophan[34], to mention a few examples. An alternative approach to hepatotoxicity is the manipulation of the cell cycle. Specifically, 2-acetaminofluorene (AAF) has been shown to inhibit hepatocyte proliferation, whilst inducing the proliferation and transdifferentiation of oval cells (hepatic progenitor cells) to hepatocytes after partial hepatectomy[35,36], thus shedding light on alternative liver regenerative pathways.

Mouse model: Although much knowledge on liver regeneration has been generated from partial hepatectomy rat models, the mouse model provides an attractive alternative due to lower costs (mice generally require fewer expensive reagents and less expensive housing)[39] relative ease of handling, and immense experimental potential afforded by genetically altered (transgenic and knockout) mice[19].

Mouse models of liver regeneration have been described in various contexts, including: partial hepatectomy[40], portal branch occlusion[41], bile duct ligation[42], chemical, pharmacological or immune-mediated injury[43-47], and chronic conditions such as non-alcoholic fatty liver disease[48] and liver cancer[49].

The lobar anatomy of the mouse and rat liver is broadly similar, and the inferior vena cava is intrahepatic in both species[39]. A significant distinction is the absence of a gallbladder in the rat[19]. In the mouse, the normal liver consists of seven lobes with the following mass proportions: left posterior (37%), left anterior (12%), right anterior (22%), right posterior (14%), right middle (8%), and two omental lobes (7%)[50]. The classical surgical model in rodents is the partial hepatectomy, which most commonly results in removal of 70% of the liver mass (also referred to as a "2/3 hepatectomy") [19]. Portal branch occlusion can be performed radiologically in humans and in large animals but requires an open surgical approach in rodents[19]. BDL has also been developed as a model of cholestasis[42], with relevance to the study of malignant biliary obstruction. Although the total BDL rat model has existed for decades, mice have been used more recently in the partial BDL model, whereby (rather than transecting the bile duct between ligatures) a 7-0 needle is ligated onto the duct. When the needle is removed, a reproducibly narrow bile duct lumen is left, which results in less liver necrosis[42] and may more closely resemble chronic cholestasis. These surgical models are of particular interest with regards to single or staged hepatec-

Table 1 Hepatotoxins used in rodent models

Toxin	Mechanism	Necrosis pattern
Acetaminophen (paracetamol)[19,36,37]	Free radical enhancement and Kupffer cell activation	Pericentral
Carbon tetrachloride[19,30,37]	Free radical enhancement and Kupffer cell activation	Pericentral
Concanavalin A[37]	T-cell activation; cytokine release; ICAM-1 & VCAM-1 upregulation.	Centrilobular
D-Galactosamine[19,37]	Uridine metabolite deficiency	Random
Ethanol[19,31]	Increases production of reactive oxygen species and infiltration of inflammatory cells	None
Lipopolysaccharide[37]	Kupffer cell activation	Centrilobular
Thioacetamide[19,37,38]	Increases production of toxic metabolites and reactive oxygen species	Pericentral

ICAM-1: Intercellular adhesion molecule 1; VCAM-1: Vascular cell adhesion molecule 1.

tomies with or without portal vein occlusion in human patients with liver tumours, where physiological reserve, oncological and technical resectability, as well as liver tumour burden and status of background parenchyma will determine the most optimal approach. However, recapitulating human liver procedures in mouse models is limited by the fact that mice are relatively very small, and (as in humans) there is significant anatomical variability in their hepatic vascular and biliary systems[39]. Furthermore, rodents can typically survive with much smaller liver remnants than humans, and the kinetics of liver regeneration vary between species. Nevertheless, surgical techniques in mice are well established and are characterized by reproducibility and minimal operative mortality[39].

The most frequently used hepatotoxins used to induce liver injury in mouse models are carbon tetrachloride (CCl_4), d-galactosamine, paracetamol (acetaminophen), ethanol[51] and thioacetamide[19]. CCl_4 can induce acute and chronic liver injury through its action on cytochrome P450, leading to the production of free peroxide radicals which cause lipid peroxidation of hepatocyte[19]. The disadvantage of the CCl_4 model is the inflammatory and immune response caused during hepatocyte injury, which may confound models of liver regeneration[19]. D-galactosamine is thought to induce liver injury *via* intracellular deficiency in uridine metabolites and can additionally induce hepatocyte apoptosis when combined with lipopolysaccharide [19]. Paracetamol is metabolized by cytochrome P450 and in overdose leads to toxic levels of N-acetyl-benzoquinone imine, free radical formation and centrilobular apoptosis/necrosis[19]. The kinetics of liver regeneration after CCl_4, D-Galactosamine and paracetamol-induced injury are similar[19]. Ethanol induces liver injury *via* mitochondrial dysfunction, oxidative stress, inflammatory cell infiltration and translocation of intestinal bacteria which can then enter the portal and systemic circulation [19]. Finally, thioacetamide leads to oxidative stress *via* its conversion to thioacetamide disulfoxide which increases the production of reactive oxygen species[19].

In addition to the hepatotoxic models mentioned above, several dietary models are used in mice to model liver disease. These include the 1,4dihydro2,4,6trimethylpyridine-3,5-dicarboxylate (DDC) diet, which leads to biliary injury and regeneration [52], the modified choline-deficient ethionine diet, which leads to hepatocellular injury, steatosis and spread of ductular cells from the portal tract[53]. More recently, a mouse model with rapid progression from normal liver to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) within 24 wk has been described by Tsuchida *et al*[49]. This was achieved by feeding C57BL/6J mice a western diet (high-fat, high-fructose and high- cholesterol) and administering weekly intraperitoneal doses of CCl_4.

The development of transgenic and knockout mouse models has enabled closer scrutiny of pathophysiological mechanisms with regards to liver regeneration after surgery or chemical/diet-induced injury, also highlighting the importance of the innate and adaptive immune system in liver regeneration[54].

The opportunities offered by these models and their relevance to the treatment of liver tumours in humans will be elaborated in the sections to follow. Table 2, whilst non-exhaustive, gives an impression of the breadth and potential of transgenic and KO mouse models in the study of liver regeneration.

Zebrafish model: Following their discovery in the Ganges River in the late 19th century, zebrafish (Danio rerio) were initially used by embryologists to investigate

Table 2 Studies of liver regeneration involving transgenic or knockout mice

Yr	First author	Gene product	Study title	Ref.
1994	Webber	TGF-α	"Overexpression of transforming growth factor-alpha causes liver enlargement and increased hepatocyte proliferation in transgenic mice"	[55]
1996	Cressman	IL-6	"Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice"	[56]
1997	Yamada	TNF	"Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor"	[57]
1998	Greenbaum	C/EBP-β	"CCAAT enhancer-binding protein beta is required for normal hepatocyte proliferation in mice after partial hepatectomy	[58]
1998	Rai	iNOS	"Impaired liver regeneration in inducible nitric oxide synthase-deficient mice"	[59]
1998	Roselli	uPA	"Liver regeneration is transiently impaired in urokinase-deficient mice"	[60]
1998	Yamada	TNFR-1TNFR-2	"Analysis of liver regeneration in mice lacking type 1 or type 2 tumor necrosis factor receptor: requirement for type 1 but not type 2 receptor"	[61]
2002	Anderson	PPAR-α	"Delayed liver regeneration in peroxisome proliferator-activated receptor-alpha-null mice"	[62]
2003	Leu	IGFBP-1	"Impaired hepatocyte DNA synthetic response posthepatectomy in insulin-like growth factor binding protein 1-deficient mice with defects in C/EBP beta and mitogen-activated protein kinase/extracellular signal-regulated kinase regulation"	[63]
2003	Strey	C3a/C5a	"The proinflammatory mediators C3a and C5a are essential for liver regeneration"	[64]
2004	Borowiak	Met	"Met provides essential signals for liver regeneration"	[65]
2004	Mohammed	TIMP3	"Abnormal TNF activity in Timp3(−/−) mice leads to chronic hepatic inflammation and failure of liver regeneration	[66]
2004	Nakamura	OSM	"Hepatocyte proliferation and tissue remodeling is impaired after liver injury in oncostatin M receptor knockout mice"	[67]
2004	Oe	TGF-β	"Intact signaling by transforming growth factor beta is not required for termination of liver regeneration in mice"	[68]
2005	Duffield	DTR	"Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair"	[69]
2005	Mitchell	HB-EGF	"Heparin-binding epidermal growth factor-like growth factor links hepatocyte priming with cell cycle progression during liver regeneration"	[70]
2005	Oliver	MT	"Impaired hepatic regeneration in metallothionein-I/II knockout mice"	[71]
2005	Seki	MyD88	"Contribution of Toll-like receptor/myeloid differentiation factor 88 signaling to murine liver regeneration"	[72]
2006	Fernández	Caveolin-1	"Caveolin-1 is essential for liver regeneration"	[73]
2006	Olle	MMP9	"Matrix metalloproteinase-9 is an important factor in hepatic regeneration after partial hepatectomy in mice"	[74]
2007	Mayoral	Caveolin-1	"Dispensability and dynamics of caveolin-1 during liver regeneration and in isolated hepatic Cells"	[75]
2009	Tumanov	Rag1LT	"T cell-derived lymphotoxin regulates liver regeneration"	[54]
2010	Erhardt	CCR5, CXCR3	"Tolerance induction in response to liver inflammation"	[47]
2010	Liu	GPC3	"Suppression of liver regeneration and hepatocyte proliferation in hepatocyte-targeted glypican 3 transgenic mice"	[76]
2012	Borude	FXR	"Hepatocyte-Specific Deletion of Farnesoid X Receptor Delays But Does Not Inhibit Liver Regeneration After Partial Hepatectomy in Mice"	[77]
2013	Bhave	GPC3	"Regulation of Liver Growth by Glypican 3, CD81, Hedgehog, and Hhex"	[78]
2014	Kong	FGF15	"Fibroblast growth factor 15 deficiency impairs liver regeneration in mice"	[79]
2014	Yang	Lrp5/6	"β-catenin signaling in murine liver zonation and regeneration: a Wnt-Wnt situation!"	[80]
2015	Lu	Mdm2	"Hepatic progenitor cells of biliary origin with liver repopulation capacity"	[81]
2016	Swiderska-Syn	Cre recombinase	"Hedgehog regulates yes-associated protein 1 in regenerating mouse liver"	[82]
2018	Tsagianni	MET	"Combined Systemic Disruption of MET and Epidermal Growth Factor Receptor Signaling Causes Liver Failure in Normal Mice"	[83]
2019	Asrud	Epac	"Mice depleted for Exchange Proteins Directly Activated by cAMP (Epac) exhibit irregular liver regeneration	[84]

			in response to partial hepatectomy"	
2019	Fortier	p38α MAPK	"Hepatospecific ablation of p38α MAPK governs liver regeneration through modulation of inflammatory response to CCl 4-induced acute injury"	[85]
2019	Modares	IL-6R	"IL-6 Trans-signaling Controls Liver Regeneration After Partial Hepatectomy"	[86]
2019	Zhou	Rictor	"Mammalian Target of Rapamycin Complex 2 Signaling Is Required for Liver Regeneration in a Cholestatic Liver Injury Murine Model"	[87]
2020	Laschinger	CGRP-RAMP1	"The CGRP receptor component RAMP1 links sensory innervation with YAP activity in the regenerating liver"	[88]
2020	Seguin	Mfrn1, Mfrn2	"The mitochondrial metal transporters mitoferrin1 and mitoferrin2 are required for liver regeneration and cell proliferation in mice"	[89]
2020	Xue	GPC3	"Phosphorylated Ezrin (Thr567) Regulates Hippo Pathway and Yes-Associated Protein (Yap) in Liver"	[90]

developmental biology[91]. Their relative low cost, rapid development from one-cell embryo to free-swimming larva 5 d post-fertilisation, optical transparency enabling direct observation using light and fluorescent microscopy, and relative genetic conservation compared to the human genome with approximately 70% of human genes having a zebrafish orthologue[92] has led to their role within medical research expanding considerably. In the realm of liver biology, applications include the study of high throughput drug discovery and hepatotoxicity screening, forward genetic screening, heritable and developmental liver diseases, the molecular and cellular factors that contribute to human liver disease, liver cancer biology and liver regeneration[91,93-95]. The research opportunities and disadvantages presented by zebrafish are summarized in Table 3.

Cell types are highly conserved between zebrafish and mammalian livers, with the exception of hepatic immune cells (Kupffer cells), which have not been identified in zebrafish. Whilst zebrafish provide immensely useful models, this difference highlights the caution needed in the extrapolation of results between species. As discussed below, Kupffer cells play an important part in cytokine priming of hepatocytes, implying that a different priming mechanism operates in zebrafish, or that this role is played by different cell type. Cellular morphology and physiology are also largely conserved with zebrafish livers demonstrating similar functions to mammalian livers including secretion of bile, glycogen and lipid storage, insulin responsiveness, ammonia metabolism and the production and secretion of proteins including complement, clotting factors and a protein resembling albumin. The morphological composition of the zebrafish, however, is distinct to the mammalian liver with the liver arranged into 3 Lobes that lack a pedicle that separates the lobes in the mammalian liver. Moreover, the portal architecture of mammalian livers is not observed. In fish, the hepatocytes are arranged into tubules with bile ductules running between two rows of hepatocytes[91].

Liver regeneration in mammalian livers involves a compensatory regeneration with hepatocyte proliferation and hypertrophy. In contrast, zebrafish demonstrate true epimorphic regeneration in response to partial hepatectomy with regrowth of the resected lobe, again highlighting significant inter-species differences. Genome-wide gene expression studies have demonstrated that liver regeneration is the result of a coordinated expression of thousands of genes, and whilst several pathways have been identified as important in liver regeneration in both mammals and zebrafish including WNT, fibroblast growth factor receptor (FGFR) and bone morphogenic protein receptor, in isolation they are insufficient to drive the complex process of liver regeneration. The mechanisms underlying the difference between mammalian liver regeneration and zebrafish, and epimorphic regeneration are still to be elucidated[96].

In vitro models

The observation of interspecies variability, the ethical guiding principles of the 3R principles (replacement, refinement, and reduction of animal testing), and the opportunity of better-defined experimental conditions have motivated the development *in vitro* models to study liver biology. Thus, two- and three-dimensional (2D and 3D) *in vitro* models have increased our understanding of the mechanisms of liver injury, hepatotoxicity, and mechanisms of liver regeneration[97].

2D culture models: 2D *in vitro* liver models, have traditionally used immortalised cell lines such as the HepG2 and the HepaRG cell lines derived from human progenitor cells[98], or mechanically and enzymatically dissociated primary cells[99] expanded on

Table 3 Advantages and disadvantages of zebrafish as a model for human liver pathophysiology

Advantages	Disadvantages
Vertebrate body plan	Partial genome duplication in teleosts
Ease of husbandry	Differences in microanatomy and liver architecture
Inexpensive to maintain	Less conserved physiology than mammalian models
Large numbers of embryos produced rapidly	Less conserved morphogenesis than mammals
External development	Less developed cell culture technology
Optical clarity during development	Poorly developed embryonic stem cell technology
Zebrafish liver not required for foetal haematopoiesis	
Amenable to forward and reverse genetics	
Molecular conservation of development	
Amenable to high-throughput screening: (1) Phenotype assessment; and (2) Drug/chemical screening	

plastic surfaces, or supported by extracellular matrix (ECM) scaffolding[100]. Though presenting advantages in terms of ease of tissue culture, such systems have limitations; for example, cells lines have fundamentally different gene expression profiles to primary hepatocytes, owing to their immortalised nature[101]. Primary hepatocytes have some benefits in this regard, but are difficult to source (in the human case), exhibit donor variability[102], and rapidly lose their differentiation and function (such as morphology and toxicant related genes expression) in plastic culture[100,103,104].

The presence of ECM partially addresses these shortcomings. Culturing primary hepatocytes between two layers of collagen, termed sandwich-cultured hepatocytes (SCH)[105], results in extended viability, retained cellular polarity with correct localization of basolateral and canalicular transporters[106] as well as formation of functional bile networks[107]. However, despite their promising properties compared to monolayer cultures, sandwich cultures have their own disadvantages including the barrier to introduced materials created by the collagen layers, and low levels of expression of cell-to-cell adhesion proteins that are critical for cell function and differentiation[108]. As such the role of sandwich culture in the experimental process is often limited to short term studies.

3D culture models: Significant progress has been made using 3D *in vitro* hepatic models with benefits in terms of maturity of hepatocytes, long term viability, and more precise representations of the microenvironment of the *in vivo* liver[109]. *In vitro* liver modelling studies with human cells have allowed investigation of liver development, liver disease modelling, liver regeneration, and therapeutic transplantation. Given the complex 3D structure and functional regionalization of the liver, 3D liver models including organoids offer the advantage of more closely recapitulating spatial organization, important cell-cell and cell-ECM contacts that stimulate proliferation, differentiation, liver specific expression, and responsiveness to exogenous stimuli[110]. These advantages have been further emphasised by the use of coculture in such systems, allowing the inclusion of key non-parenchymal cells[97].

Human-induced pluripotent stem cells (hIPSCs) offer an effectively unlimited source of genetically diverse cell lines that can be generated from both healthy and diseased livers. Furthermore, these cells are amenable to genetic modification using the CRISPR technology in order to facilitate disease modelling[111]. hIPSCs have further expanded the opportunity for 3D *in vitro* culture systems by the development of hepatic organoids from hIPSCs[112]. Thus, reports describe the design of organoids involving multiple cell types by co-differentiating hepatocytes and cholangiocytes [113], or hepatocytes with other supporting cell types including stellate-like and Kupffer-like cells[114]. However, an important limitation is the relative immaturity of hepatocyte-like cells generated from hIPSCs. This is demonstrated by continued alpha-fetoprotein (αFP), low albumin expression, and distinctive CYP expression and function[115]. The problem of functional maturity has been partially addressed by modifying culture conditions, including the medium composition (*e.g.*, inclusion of specific growth factors, hormones)[115]. More recent approaches to circumvent the disadvantages of hIPSCs have involved the use of primary cells to form organoids.

Thus, Huch *et al*[116] generated human liver organoids from primary ductal Epidermal Cell Adhesion Molecule positive cells grown in a defined human liver media allowing culture with stable function for over 6 mo, and Justin *et al*[117] describe the formation of biliary organoids from primary cholangiocytes.

In spite of these advances, 3D *ex vivo* cultures do carry their own drawback including difficulties in controlling cellular distribution, innervation, and vascularization-with the latter aspect of particular importance given the central role of the liver sinusoids to hepatic function.

Repopulation of decellularised livers: Repopulation of decellularised liver scaffolds with cells has offered a further refinement to the *in vitro* investigation of liver regeneration as well as potential therapeutic opportunities[118].

Earlier approaches to generating functional livers include hepatocyte transplantation in humans[119], as well as in animals[120]. In the latter case, microcarriers and biodegradable polymer scaffolds have been described, resulting in albumin production and clearance of bilirubin and urea metabolites[120]. These efforts have laid the ground for three-dimensional scaffolds[121] which are either biological membranes[122], collagen sponges[123], or synthetic hydrogels[124], and which enable the production of hepatic organoids. In another approach to liver regeneration, chimeric murine models have been developed, whereby mouse liver is extensively repopulated with human hepatocytes, thus permitting the study of liver disease (*e.g.*, viral hepatitis) within humanised organs *in vivo*[125].

More recently, techniques in whole liver decellularization and repopulation have moved the field further, although significant challenges remain. In general terms, the process involves removal of the liver's cellular and immunogenic components, thus creating a scaffold which retains the ultrastructure and properties of the ECM[126]. This is usually followed by static cultivation of cells (*e.g.*, hepatocytes) and their subsequent infusion into the scaffold.

In one of the first such studies, Uygun *et al*[127] demonstrated that ischaemic rat livers can be decellularised whilst preserving structural and basement-membrane-based components of the ECM, as well as the microvasculature. The investigators achieved decellularisation by portal vein perfusion using sodium dodecylsulfate (an anionic detergent), and repopulation with primary rat hepatocytes *via* the same route. Recellularised grafts were implanted in rats for 8 h, and after explantation underwent *ex vivo* blood perfusion for 24 h, demonstrating ongoing hepatocyte metabolic activity. Others have demonstrated that implanting repopulated ECM liver scaffolds into rats which had undergone extended hepatectomy improved liver function and extended their mean lifespan from 16 to 72 h[128]. In the last 10 years, a variety of animal models, decellularisation techniques, repopulation routes and cell sources have been described, with promising outcomes in terms of vascular repopulation[118,129,130], hepatocyte survival[131] as well as formation of biliary duct-like structures and activation of liver detoxification enzymes[132]. One of the commonest sources of liver scaffolds is the rat[118,127,132-137] repopulated with rat hepatocytes (although cholangiocytes[136] and lineages from pluripotent stem cells, mesenchymal cells, and fibroblasts have also been described[137] usually *via* the portal vein. With regards to human tissue, Verstegen *et al*[138] demonstrated that decellularised human livers can be repopulated with human umbilical vein endothelial cells, leading to re-endothelialisation of the vascular tree. Table 4 presents further examples of the different approaches to liver decellularisation-repopulation developed thus far.

The main challenges in producing a viable whole organ from liver decellularisation-repopulation techniques include heterogeneity of cell engraftment, thrombosis (partly related to incomplete or suboptimally functional endothelium as well as microvascular injury[121,130]), the re-creation of an intact and functional biliary tree, as well as attaining the specific distribution of liver cell types seen in the native healthy organ. Mesenchymal and pluripotent stem cells for repopulation are currently considered attractive research avenues[121] as they may lead to more clinically applicable models.

Human models

The study of human liver regeneration is limited to observational data in the context of clinical pathology and applied therapies, and thus contrasts to the directed experimental approaches possible in animal models. Moreover, access to human liver tissue during the regenerative process is not possible as liver biopsy can only be justified by clinical need, given the risks of the procedure including a measurable mortality[139]. The available observational data comes from a combination of clinical findings, serum biomarker measurements, and imaging. Clinical observations and blood bio markers are subject to difficult interpretation because of the confounding effects of the hetero-

Table 4 Examples of liver decellularization-repopulation studies

First author	Yr	Liver scaffold source	Cell source & type	Repopulation route	Outcomes	Ref.
Uygun	2010	Rat	Rat hepatocytes	Portal vein	Recellularised liver grafts implanted in rats, perfused in vivo for 8 h, explanted and assessed after 24 h, demonstrating hepatocyte survival, albumin secretion, urea synthesis and cytochrome P450 expression.	Uygun 2010 [127]
Zhou	2011	Mouse	Human foetal hepatocytes	Portal vein	Recellularised liver matrix implanted in mice, achieving hepatocyte survival after 6 wk, with albumin secretion and cytochrome P450 expression.	Zhou 2011 [131]
Ko	2014	Pig	Murine endothelial cells, after scaffold conjugation with rat anti-mouse CD31 antibodies	Portal veinHepatic arteryInferior vena cava	Recellularised liver grafts implanted in pigs, demonstrating good blood flow and patency throughout vascular network over 24 h after transplantation.	Ko 2015 [130]
Navarro-Tableros	2015	Rat	Human liver stem-like cells	Portal vein	Loss of embryonic markers, expression of albumin, lactate dehydrogenase and cytochrome P450 subtypes. Production of urea and nitrogen.	Navarro-Tableros 2015[133]
Ogiso	2016	Rat	Mouse hepatocytes	Biliary tree; Portal vein	(1) > 80% of cells seeded *via* biliary tree entered the parenchyma; (2) Approximate 20% of cells seeded *via* portal vein entered the parenchyma; and (3) Increased gene expression of foetal hepatocyte albumin, glucose 6-phosphatase, transferrin, cytokeratin 19, and gamma-glutamyl transpeptidase, activation of liver detoxification enzymes, formation of biliary duct-like structures.	Ogiso 2016 [132] [PMID 27767181]
Verstegen	2017	Human	Human umbilical vein endothelial cells.	-	Re-endothelialisation of vascular tree, demonstrated by luminal vimentin and von Willebrand Factor/F8 staining.	Verstegen 2017[138]
Butter	2018	Rat	Rat hepatocytes	Hepatic artery and portal vein	In vitro demonstration of hepatocyte spread to all liver lobes, with proliferation, and production of aminotransferases, lactate dehydrogenase and albumin.	Butter 2018 [134]
Chen	2018	Rat	Rat hepatocytes	Portal vein	None (description of materials and methods).	Chen 2018 [135]
Chen	2019	Rat	Rat cholangiocytes Rat hepatocytes	Common bile duct; Portal vein	In vitro viability and function demonstrated by albumin and urea secretion, and gene expression of functional proteins.	Chen 2019 [136]
Harper	2020	Rat	Rat bone marrow cells	Portal vein	Stem cells engrafted in portal, sinusoidal and hepatic vein compartments, achieving expression of endothelial cell surface markers for up to 30 d.	Harper 2020 [118]
Takeishi	2020	Rat	Human hepatocytes, biliary epithelial cells, and vascular endothelial cells derived from pluripotent stem cells, mesenchymal cells, and fibroblasts.	Biliary tree; Portal vein; Central veins	Auxiliary grafts implanted in rats, achieving in vivo functionality for 4 d.	Takeishi 2020[137]

geneity of the study population, diverse pathologies, and varied clinical scenarios even within a defined patient group. Although there are reports of serum biomarkers such as αFP and micro RNAs correlating with liver regeneration, their clinical applicability remains to be established. Combining clinical and serological measurements, scores such as the Acute Liver Failure Study Group index has allowed the identification of patients likely to require liver transplant[140-142].

In this context, the relatively non-invasive nature of modern imaging techniques has provided the main means of assessing liver growth and function, as markers of regeneration. Although liver function correlates well with liver volume in uncompromised livers, this relationship is less clear in patients with pre-existing parenchymal liver[143,144]. Estimation of remnant liver function instead of remnant liver volume is a better predictor of clinical outcome after liver resection in patients with decreased liver function[145]. In order to avoid PHLF, clinicians must ensure that the future remnant liver (FRL) will be sufficient to sustain life. Traditionally, this functionality assessment is made by pre-operatively measuring the volume of the FRL as a surrogate measure of functionality[144]. Volumetry, however, assumes liver parenchymal homogeneity and normal underlying liver function, which are not always present in patients undergoing extensive hepatic resections. This lack of

homogeneity in hepatic function can cause a discrepancy between FRL volume (FRL-V%) and FRL function (FRL-F)[146] which is especially important in patients who present with pre-existing liver disease or who have previously received chemotherapy that resulted in steatotic or microvascular liver changes[146]. As such, FRL-V% cut-off values may not accurately predict the quality of the FRL in some patients, with implication on the development of PHLF and associated mortality. The radiological modalities most used to predict the FLR are outlined below.

Standard liver volumes can be calculated from the patient's body surface area or mass using the formulas originally proposed by Vauthey *et al*[144]. However, these formulas are limited by subject demographics (healthy individuals) and by their modest correlation to liver sizes calculated by more advanced forms of volumetry [147]. CT volumetry of the liver was first performed on cadavers by Heymsfield *et al* [148] in 1979 and was shown to be accurate within 5% of water displacement volumetry. CT is more commonly used due to its greater accessibility, higher spatial resolution, and short acquisition time. MRI, conversely, offers multiple contrast mechanisms and the ability to assess vascular and biliary anatomy in addition to parenchymal pathology. Additionally, MRI also minimises the risk of contrast induced nephrotoxicity and eliminates concerns of radiation exposure[149]. Liver segmentation has emerged as the preferred technique CT volumetry can be used to calculate the volume of the FLR and is widely used to exclude patients from liver resection or to select patients who will benefit from a procedure to increase the volume of the future remnant, such as PVE[150]. However, the outcomes of previous reports correlating the findings of CT volumetric analysis of the future remnant with post-resectional outcome, have not been consistent and the role alternative imaging modalities has been examined[150,151].

Hepatobiliary scintigraphy using 99mTc-iminodiacetic acid analogues, such as 99mTc-mebrofenin, can be used to measure segmental liver function. 99mTc-mebrofenin is excreted into the bile by adenosine triphosphate–dependent export pumps the multidrug-resistance-associated proteins 1 and 2 without undergoing biotransformation during transit through the hepatocytes[146,152,153] . Previous reports in the literature have shown that 99mTc-mebrofenin hepatobiliary scintigraphy (HBS) can provide clinicians with information on FRL-F instead of volumetric information alone[153]. HBS provides visual and quantitative information of global and regional liver function as well as excretory function (intrahepatic and extrahepatic bile transport). 99mTc-mebrofenin is intravenously injected and consequently excreted in bile by the hepatocytes without undergoing biotransformation. As such, the clearance measurement of Technetium-99m mebrofenin using scintigraphy can quantify hepatic function[146,153]. FRL-F assessment using HBS has been proven to be superior to volumetry in the prediction of PHLF and PHLF(M), making HBS the imaging modality of choice prior to proceeding with major hepatectomy. Reports in the literature have illustrated that an HBS cut-off value of 2.7%/min/m^2 can outperform volumetry cut-off values in the prediction and prevention of PHLF and PHLF(M) by identifying high-risk patients with borderline predicted remnant liver function, and consequent selection for pre-operative PVE or other hypertrophic strategies (*e.g.*, ALPPS[154,155]. Certain hepatobiliary units have already implemented HBS in favor of CT volumetry before hepatic resection based on emerging evidence in the literature.

SECTION 2: EARLY EVENTS POST LIVER INJURY AND TRIGGERS TO LIVER REGENERATION

The PH model in rodents has allowed the examination of immediate events which occur within minutes of liver resection and provides an insight into the mechanisms that trigger the process of liver regeneration. These early events relate to vascular portal flow, tissue hypoxia, haemostatic mechanisms, and changes in extracellular matrix integrity.

Vascular events

Following PH, the increased portal blood flow through the remnant liver exerts a heightened shear stress on the LSECs[156]. Shear stress on LSEC induces numerous physiological changes[157] including microscopically visible ones such as increased sinusoidal diameter and changes to LSEC fenestrae and sieve plates[158,159]. Shear stress also induces biochemical responses including the release of vascular endothelial cell growth factor (VEGF) from LSEC[160], the secretion of VEGF and HGF from

hepatic stellate cells[161], and the LSEC production of nitric oxide (NO) by Nitric Oxide synthase (NOS), which increases hepatocyte sensitivity to HGF[162,163]. The physiological importance of NO is suggested by the finding that inhibition of NOS severely impairs liver regeneration in mice after PH[59].

Shear stress also induces the hepatocyte priming cytokine interleukin 6 (IL6) in LSEC[164], as well as expression in of liver regeneration associated WNT, VEGF, and epithelial cell adhesion molecules in hepatic progenitor cells[165].

Another consequence of increased portal flow through the remnant liver is increased exposure to lipopolysaccharide (LPS), which is derived from gut bacteria, and which translocates from the gut into portal blood. PH increases the concentration of LPS in the remnant liver not only because of diversion of more portal blood to the remnant liver, but also because the rise in portal pressure increases intestinal permeability, allowing greater LPS translocation[166,167]. In the sinusoids, LPS binds Toll like receptors (TLR) on Kupffer cells, resulting in the secretion of the hepatocyte priming cytokines IL6 and tumour necrosis factor alpha TNFα[168], in a signalling pathway that is dependent on myeloid differentiation factor 88[169].

The increased expression of liver regeneration promoting biochemicals is not confined to the liver. Following PH in the rat, increased expression of VEGF, HGF, and hypoxia inducible factor (HIF) is also observed portal vein drained tissues such as the spleen and small intestine, whereupon portal VEGF concentrations exceed those of the systemic circulation. The mechanism stimulating this extrahepatic expression of growth factors from portal drained tissues is unclear but may also be related to portal pressure changes[170].

Hypoxia

Following PH, increased portal flow brings about a reflex arterial vasoconstriction (the arterial buffer response), which can result in hypoxia in the remnant liver, given the low partial pressure of oxygen (pO2) in portal venous blood[171].

An important outcome of hypoxia is the induction of HIF, which in turn leads to the activation of multiple genes involved in tissue adaptations to hypoxia ranging from glycolytic metabolism to angiogenesis[172].

In the liver, PH leads to increased expression of HIF and subsequently VEGF[173]. In elegant experiments, Dirscherl *et al*[174] show that the hypoxic environment triggers hepatic stellate cell expression of HIF, resulting in increased expression of VEGF, which then elicits a range of responses in LSEC including proliferation and angiogenesis, but also genes associated with matrix remodelling (discussed in a later section), and LSEC expression of the potent hepatocyte mitogen HGF[174], as well as other liver regenerative genes[175]. Thus, the authors suggest that HSC function as hypoxia sensors in the liver, and trigger angiogenesis in liver regeneration, highlighting the complexity of intercellular cross-talk in this process. In addition, hypoxia induced secretion of complex regeneration promoting molecules from stem cells at extra-hepatic locations may also contribute to promotion of liver regeneration [176].

Haemostasis related factors

The injury to liver tissue in PH results in the activation of mechanisms for haemostasis carried out by platelets and the coagulation cascade. The role of platelets is not confined to haemostasis, but also includes functions relating to liver regeneration [177], and studies in animals and humans suggest impaired liver regeneration in individuals with low platelet counts[178,179].

Following PH, platelets migrate to the space of Disse, where they release liver regeneration promoting biochemical including serotonin, VEGF, and HGF from secreted cytoplasmic granules[180]. In addition to growth factor containing vesicles, platelets contain cytoplasmic RNA, which can be transferred to nearby hepatocytes, resulting in gene expression, and promoting hepatocyte proliferation[181]. Finally, platelets may stimulate liver regeneration by activation of immune cells which also have an important role in cellular cross-talk[180].

In addition to the role of platelets, the coagulation cascade both individually and in combination with 'damage associated molecular patterns' (DAMPs) (including mitochondrial DNA and peptides)[182], activates elements of the complement cascade [183]. These include C3a and C5a, which have a role in stimulating pathways involved in the priming of hepatocytes[184], enabling them to respond to growth factors, as discussed in "*Priming of hepatocytes*".

Other elements of the coagulation cascade may also play key roles in liver regeneration. Thus, Groeneveld *et al*[185] report that intrahepatic deposition of fibrinogen after PH is a key driver to platelet accumulation in the liver. Fibrinogen

depletion was associated with impaired liver regeneration in a mouse model, and in humans undergoing liver resection, low intrahepatic fibrinogen and low post op serum fibrinogen levels were associated with poor liver function an increased mortality.

ECM changes

Urokinase-type plasminogen activator (uPA) activity increases within one minute of PH in rats[186], resulting in the activation of plasminogen to plasmin, which then activates key metalloproteinases (MMP) such as MMP-9[187,188], which remodel the hepatic ECM, where HGF is present in its inactive form. uPA also activates HGF to its active form[189], releasing it locally in the liver parenchyma and also into the circulation in significant quantities[190]. uPA knockout mice show impaired liver regeneration[60]. As well as HGF, the ECM contains other inactive forms of growth factors including HB-EGF and fibroblast growth factor (FGF)[191]. Moreover, the importance of matrix alteration in the initiation goes beyond the release of growth factor stores in the ECM in that hepatocyte response to key growth factors is ineffective in the presence of intact ECM, and that ECM changes are required for growth factor driven hepatocyte proliferation[192,193].

Thus, the rapid action of uPA following PH provides a mechanism to kick start the liver regenerative process by liberating ECM stored growth factors, until such time as other mechanisms begin to contribute to maintaining the liver regenerative process.

SECTION 3: HEPATIC MITOGENS

Liver regeneration is characterized by hypertrophy and rapid proliferation allowing return to the starting volume of liver even if recovering from a 25% remnant in humans, or a 10% remnant in some rodent models. The proliferation of hepatocytes is controlled by a maelstrom of growth factors with different but overlapping effects. Within this complexity exists a hierarchy of functions, whereby hepatocytes first require to be primed ("*Priming of hepatocytes*"), after which they become responsive to a range of mitogens referred to as complete ("*Complete mitogens*") auxiliary ("*Auxiliary mitogens*"), and complex ("*Complex mitogens*"). "*Intracellular signalling pathways*" summarizes the intracellular pathways which transmit the effect of the growth factors described in sections "*Priming of hepatocytes*" - "*Complex mitogens*".

Priming of hepatocytes

Hepatocyte transition from G0 to G1: Although proliferation of hepatocytes is stimulated by a wide range of biochemicals in response to injury, most hepatocytes in uninjured liver do not proliferate[194], although there is some heterogeneity in this regard as discussed in "*Hepatocyte response heterogeneity after PH*" entitled 'hepatocyte regenerative heterogeneity'. The stimulus to proliferation from the multitude of mitogens requires hepatocytes to be 'primed', a complex phenomenon characterized by the induction of > 100 genes[195], which then enables the hepatocytes to respond to these mitogenic stimuli.

Although cell cycle biology is outside the scope of this review, a brief summary of key events is useful to frame the subsequent sections relating to the priming effects of cytokines and proliferative stimulus of mitogens on hepatocytes.

The cell cycle is divided into 2 main phases: mitosis (the actual process of cell division) and interphase (the phase preparing the cell for mitosis). Interphase is further divided into 3 stages, which, in order, consist of the G1 phase (during which the cell synthesises protein and organelles), S phase (during which DNA is replicated) and G2 phase (during which the machinery for mitosis is assembled). Although some cells undergo this cycle continuously, others exit the cycle and enter a stationary phase G0. In order for a cell in G0 to replicate, it first needs to be 'primed' by molecular signals to return to G1, whereupon a different set of signals will determine the speed of replication and how long it continues.

Hepatocytes provide an example of this situation, and are, in the absence of injury, almost entirely in G0[194]. Their proliferation therefore requires priming factors to return them to G1. The priming function is carried out by cytokines TNFα and IL6.

Thus the current working model[184] (illustrated in Figure 1) suggests that the cytokine priming mechanism starts with the activation of nuclear factor-kappa B (NFκB) in Kupffer cells. NFκB activation may be triggered by several stimuli including (1) Binding of TNFα to its receptor; (2) Binding of complement components C3a & C5a to their receptor; or (3) Binding of lipo-polysaccharide to the TLR receptor. Activation

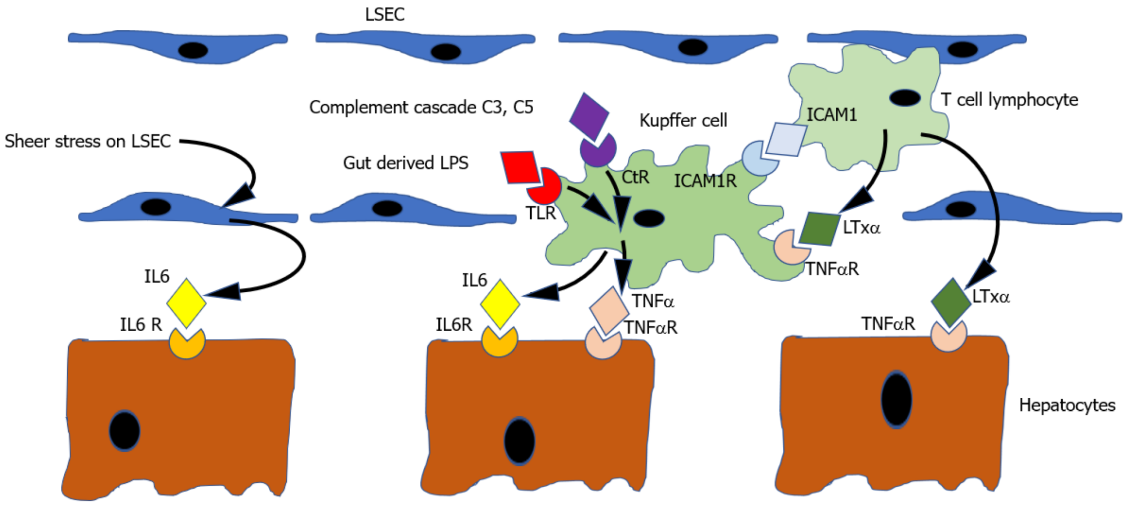


Figure 1 Cytokine priming of hepatocytes. PH induced increase in portal pressure exerts sheer stress on LSEC inducing IL6 secretion. Gut derived LPS, complement components C3 & C5, ICAM1, and LTXα from T lymphocytes all induce IL6 and TNF expression from Kupffer cells. IL6 & TNFα prime hepatocytes after binding to IL6R and TNFαR. LTXα also acts directly on hepatocytes *via* the TNFαR. LSEC: Liver sinusoidal endothelial cell; IL6: Interleukin 6; IL6R: Interleukin 6 receptor; LPS: Lipopolysaccharide; TLR: Toll-like receptor; TNFα: TNF alpha; TNFαR: TNF alpha receptor; CtR: Complement receptor; ICAM1: Intercellular adhesion molecule 1; ICAM1R: Intercellular adhesion molecule 1 receptor; LTXα: Lymphotoxin alpha; LTXαR: Lymphotoxin alpha receptor.

of NFκB results in increased expression of both TNFα and IL6. TNFα may stimulate its own further expression in the Kupfer cell in an autocrine manner. IL6 binds IL6R on hepatocytes, producing activation of signal transducer and activator of transcription 3 (STAT3), which results in the transcription of multiple other genes which push hepatocyte from G0 to G1, thus priming the cell to be responsive to circulating growth factors.

Crucially, *in vivo*, infusion of the powerful complete mitogens EGF and HGF produces only modest hepatocyte proliferation, whereas marked hepatocyte proliferation is observed if EGF and HGF infusion is preceded by the priming effect of a single TNFα injection[196].

Consistent with this model the following events are observed in the minutes after PH: (1) TNFα and IL6 mRNA and protein increase immediately[197,198]; and (2) Activation of the transcription factors NFκB and STAT3[199,200]. Moreover, DNA replication in hepatocytes is blocked by TNFα antibodies[201], TNF receptor (TNFR)[57] and IL6[56] knockout mice show impaired liver regeneration, and liver regeneration in TNFR knockout mice is rescued with IL6 infusion[57].

Of note, highlighting the necessary caution needed before extrapolating between animal models, TNFα levels after PH differ between rats and mice, with higher levels in rats. Also, the model exemplifies the recurring theme of redundancy in the system with the TNFα knockout mice showing normal liver regeneration because of the ability of other ligands to bind the TNFR[202]. Similarly, the activation of STAT3 may be achieved by other cytokines than IL6, such as Stem Cell Factor[203] and Oncostatin [203].

Triggers to cytokine priming: The initial triggers to expression of the priming cytokines TNFα and IL6 after PH are doubtless numerous and not all identified, but at least 5 stimuli have been demonstrated.

Firstly, PH results in an immediate increase in portal venous pressure which causes a sheer stress on liver sinusoidal endothelial cells[156]. This physical stimulus has many consequences[159] which are discussed in more detail in section 2 on early events post hepatectomy, but which include the induction of IL6 expression in LSECs [164], thereby contributing to the priming of hepatocytes .

Secondly, another trigger to cytokine expression after PH is binding of LPS derived from gut bacteria and translocated to portal blood, to the TLR, and producing expression of IL6 and TNFα. The increase in portal pressure resulting from PH increases gut permeability and may therefore result in exposure of the remnant liver to higher concentrations of LPS[204]. Supporting the physiological relevance of this hypothesis, is the observation that rodents with germ free guts have impaired liver regeneration[205]. The effects of LPS on liver regeneration may not be limited to induction of the priming molecules IL6 and TNFα, but also producing an increase in secretion of hepatic mitogens including insulin, epidermal growth factor, and triiodo-

thyronine[204] (see "*Complete mitogens*" and "*Auxiliary mitogens*" on complete and auxiliary mitogens).

Thirdly, it is also known that binding of complement cascade components C3 and C5 to the complement receptors on Kupfer cells also triggers an NFκB dependant increase in both IL6 and TNF. Thus, complement activation resulting from physical injury to liver in PH may also contribute to the initiation of cytokine priming of hepatocytes. The significance of this mechanism is suggested by the finding that following PH, C3-5 knockout mice show diminished activation of NFκB and STAT3, decreased expression of TNFα and IL6 impaired liver regeneration[64].

Fourthly, it is observed that mice lacking the receptor intercellular cell adhesion molecule 1 (ICAM1) show diminished TNFα and IL6 expression and impaired liver regeneration after PH. It is thought that leucocytes, attracted to a liver injury site may mediate triggering of ICAM 1 on Kupfer cells, thus providing another stimulus to initiating the cytokine cascade[206].

Fifth, it is known that the TNFR may be activated not only by TNFα, but also by the protein lymphotoxin alpha (LTxα). This is markedly upregulated in intra-hepatic T lymphocytes after PH[207] and may thus allow T cells to contribute to initiation of the cytokine cascade by activation of TNFR on Kupffer cells. Consistent with this, mice lacking both TNFα and LTα show impaired liver regeneration[208]. Moreover, LTxα may act directly on hepatocytes.

Thus, having been primed by the initial injury triggered cytokine cascade, hepatocytes return to the G1 phase of the cell cycle where they are susceptible to stimulation by mitogens including growth factors, hormones and other biochemicals to accelerate the rate of proliferation.

The concept of mitogen hierarchy: A multitude of different hepatocyte mitogens have been identified which originate from a variety of different tissues, different cell types within a given tissue, acting *via* different receptors, or sometimes overlapping in their receptor binding, and producing a variety of different effects on the target hepatocyte. This complexity exemplifies a key feature of liver regeneration biology, which is the existence of high levels of redundancy, presumably an evolutionary outcome enabling the liver to cope meet the wide range of physical, biochemical and infectious injuries it may encounter.

Amongst this complexity however, as arisen the concept of a hierarchy amongst hepatic mitogens, classifying them as 'complete mitogens', 'auxiliary mitogens', and 'complex mitogens'[209]: (1) Complete mitogens cause proliferation of hepatocyte cultures in serum- free media, and, when injected into whole animals, cause liver enlargement and hepatocyte DNA synthesis. Moreover, ablation of both the MET and EGFR pathways leads to complete inhibition of liver regeneration. The complete mitogens are (a) Hepatocyte growth factor which binds to its receptor MET; and (b) Ligands of the EGFR: EGF, transforming growth factor-α (TGFα), heparin- binding EGF- like growth factor and amphiregulin; (2) Auxiliary mitogens do not cause hepatocyte proliferation in culture in serum free media, do not cause hepatocyte DNA synthesis and liver enlargement when injected *in vivo*, and ablation of their signalling pathways delays but does not abolish liver regeneration. The auxiliary mitogens are noradrenaline and the α1- adrenergic receptor, VEGF and its receptors (VEGFR1 and VEGFR2), bile acids, serotonin, insulin, and growth hormone; and (3) Complex mitogens are the third category and are much less well defined than complete or auxiliary mitogens, with pathways involving multiple overlapping extracellular signals, disruption of which delays but does not abolish liver regeneration. The complex mitogens are the proteins involved in the *Wnt*, *β-catenin*, *Hippo* and *Yap* pathways.

Complete mitogens

Hepatocyte growth factor: HGF was the first complete hepatic mitogen, identified in 1984 with the human homolog cloned in 1989[210]. Thus, HGF produces hepatocyte proliferation in serum free media *in vitro*, and liver enlargement when infused *in vivo*. HGF mediates its effect on hepatocytes by binding to its receptor MET, a receptor tyrosine kinase with wide ranging roles in diverse areas of cell biology including not only cell survival and proliferation[211], but also metabolism[212], growth and development[213]. MET signalling is dependent on the transcription factor CCAAT/enhancer-binding protein beta C/EBP beta[214], and Inhibition of MET signalling results in blocking of mitosis and increased expression of apoptosis genes after PH[215].

After PH, HGF is mobilised in a biphasic manner, first with the activation and recruitment of ECM bound inactive HGF in the immediate minutes after PH, and then secondly by secretion of newly expressed HGF in a second wave.

Thus, whilst HGF is bound in inactive form in the ECM in resting liver[216], ECM remodelling[187] resulting from PH results in activation of HGF with binding to hepatocytes and released into the circulation[217], which peaks 30-60 min after PH.

Thereafter, peaking at 24 h post PH[218], a second wave of HGF is observed, newly synthesized by LSEC and stellate cells in the liver, but also from extra-hepatic cells and tissues including platelets[219], lung[220], kidney, spleen[221], thyroid, brain, and salivary glands[221]. In spite of these multiple sites of HGF production, experiments using genetically altered mice showed that inhibiting HGF production specifically in LSECs resulted in impaired liver regeneration, suggesting that extra-hepatic HGF production cannot compensate for depletion of hepatic HGF production[222]. The factors that stimulate HGF expression in the second wave after its release from ECM include noradrenergic signals[223], insulin like growth factors[224].

Epidermal growth factor: Ligands of the EGFR make up the other known complete mitogens. EGF is one member of a family of 7 Ligands which bind a group of 4 receptors (EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4)[225]. Of the 7 known ligands, the ones that relate to liver regeneration are epidermal EGF, transforming growth factor-alpha (TGFα), Amphiregulin (AR), and Heparin bound EGF-like growth factor (HB-EGF), with their role illustrated in Figure 2.

EGF is a complete mitogen and produces hepatocyte proliferation *in vitro* and *in vivo* when infused[226]. It is produced in many tissues[227], but the most relevant sites of production are the Brunner glands of the duodenum which provide a constant supply of EGF to the liver *via* the portal vein[228]. EGF production is increased by noradrenaline which is secreted during the physical stress of PH[229]. EGF is also produced in significant quantities in salivary glands, and sialadenectomised rats do show impaired liver regeneration after hepatectomy[230].

TGFα is produced by hepatocytes themselves during liver regeneration[231] and therefore functions as a mitogen in an autocrine or paracrine way[232]. TGFα knockout mice have no liver regeneration deficiency however, presumably as a result of the considerable redundancy in the EGFR signalling pathway[233].

Amphiregulin, like TGFα, is produced by hepatocytes. Its expression is in part regulated by inflammatory mediators providing a mechanism for its upregulation following PH. Its significance is suggested by the observation that AR knockout mice have impaired liver regeneration[234,235].

HB EGF is produced Kupffer cells and sinusoidal endothelial cells[236]. Its expression seems to be in part determined by the magnitude of liver resection, as it is increased in 2/3 PH but not 1/3 PH. Its physiological significance is emphasised by the fact that HBEGF transgenic mice[237] and HB EGF knockout mice[70] have accelerated and delayed liver regeneration, respectively.

In the midst of these multiple ligand binding events, EGFR activation peaks at 60 minutes post PH[238], and ablation of EGFR by antisense RNA impairs liver regeneration[239].

Auxiliary mitogens

Bile acids: Primary bile acids are synthesized in the liver by a multistep oxidative metabolism of cholesterol and secreted in bile. In the intestine, bile acids emulsify fats thus facilitating their digestion. Bile acids are metabolized by gut bacteria to produce secondary bile acids, and although some are lost through faecal excretion, a significant proportion are reabsorbed in the gut and recycled in the liver, in the entero-hepatic circulation[240].

Above a certain concentration, bile acids are toxic to liver and may induce apoptosis and necrosis, such that bile salt synthesis is tightly regulated by means of negative feedback loops involving bile acid receptors in the ileum[241]. At non-toxic concentrations however, bile acids play an important part in regulation of liver regeneration. Both the negative feedback controls and liver regenerative roles are mediated by bile acid receptors which comprise the extracellular TGR5 receptor (TGR5) on Kupffer cells [242], and intracellular Farnesoid X receptor (FXR) within hepatocytes[243].

Bile acids were first investigated as candidate factors for controlling liver regeneration in part because of their exclusively hepatic synthesis, offering the potential for a feedback loop hepatostatic mechanism. Thus, dietary bile acid supplementation was found to produce hepatomegaly in mice with non-injured livers, and increase liver regeneration after PH, in an effect that was dependent on the FXR. Conversely, bile acid sequestering agents resulted in impaired liver regeneration[243].

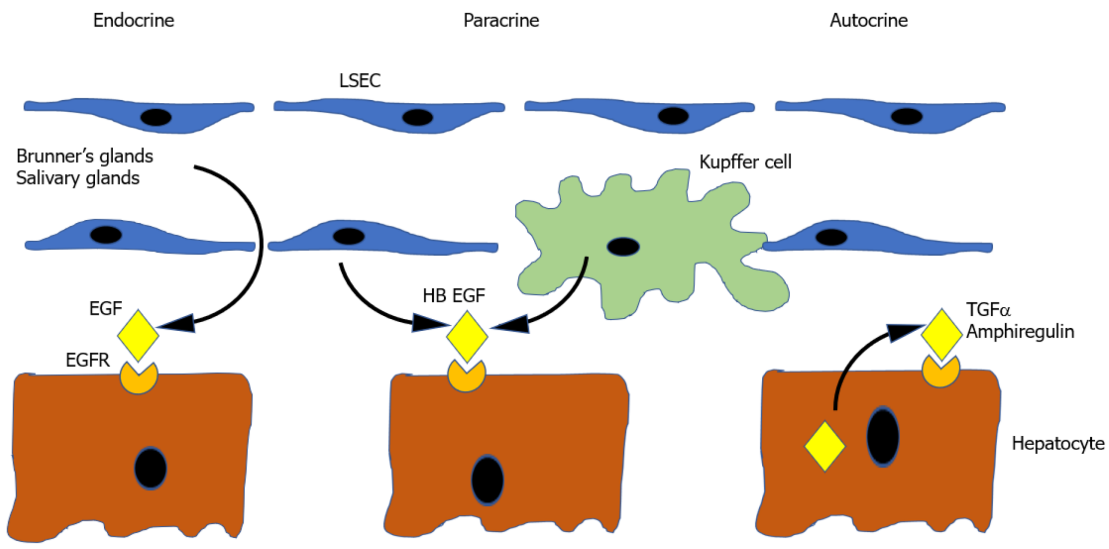


Figure 2 Summary of ligand binding to epidermal growth factor receptor in liver regeneration. Endocrine EGFR signalling by EGF from Brunner's glands and salivary glands. Paracrine EGFR signalling by HB EGF from LSEC and Kupffer cells, autocrine EGFR signalling by amphiregulin and TGFα from hepatocytes. LSEC: Liver sinusoidal endothelial cell; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; HB EGF: Heparin bound EGF-like growth factor; TGFα: Transforming growth factor α.

Furthermore, genetically engineered bile salt deficient mice also show impaired liver regeneration after PH[244], and rats having undergone PH with biliary fistula also show impaired liver regeneration, which can be rescued by intestinal delivery of bile acids[245].

After PH, serum bile acid concentration increases in blood within minutes, peaks at 24 h, and diminishes again by 48 h. The mechanism of this serum bile acid increase is not fully understood, but may involve neurological pathways activated by PH related changes in portal pressure[246], consistent with the observation that bile acid increase is also seen after portal vein embolization[247].

The binding of bile acids to the FXR stimulates activation of transcription factor Forkhead box M1b (FoxM1b), an injury-induced transcription factor that promotes cell cycle progression[248]. In addition, bile acids also contribute to liver regeneration by binding extra-hepatic FXR situated in the ileum, resulting in expression of fibroblast growth factor (Fgf15/FGF19). Fgf15/FGF19, which then binds it receptor FGFR4[249] on hepatocytes, stimulating cell cycle progression[241]. In comparison to the FXR receptor, the role of bile acid binding to the TGR5 receptor on Kupffer cells is less well understood, but is clearly important as TGR5 knockout mice show impaired liver regeneration after PH, as well as severe hepatic necrosis[250].

Thus, bile acids have an important role in the control of liver regeneration and may contribute to the post liver injury hepatostat.

Noradrenaline: Noradrenaline secretion increases following PH[251] and is produced by the adrenal medulla, sympathetic neurons, as well as by hepatic stellate cells.

Noradrenalin not only stimulates the production of EGF (from Brunner's glands) and HGF from fibroblasts, but also augments their mitogenic effect[252], and activates the proliferation associated STAT3 pathway[253], whilst reducing the mito-inhibitory effects of TGFβ[254]. Thus, α1 receptor blockade, and also hepatic sympathectomy significantly delays liver regeneration after PH[251]. Noradrenaline may also stimulate liver regeneration by activating WNT and β-catenin pathways *via* β-adrenergic receptors[255].

Serotonin: Serotonin is a neurotransmitter stored by platelets and which has a role in the control of inflammation. Mice with absent platelets or lacking tryptophan hydroxylase 1 (a key enzyme in serotonin synthesis) show significantly delayed liver regeneration after PH[256], which is rescued by serotonin infusion. Moreover, serotonin agonist produces LSEC fenestration changes, and a VEGF dependent increase in hepatocyte proliferation[257]. Serotonin may also act *via* the Hippo proliferative pathway[258]. Although serotonin deficient mice show significantly impaired liver regeneration, serotonin exemplifies the need for caution in assuming that the results of one animal model may be extrapolated to others, as it is noted that rats

lacking the serotonin transporter which are unable to store serotonin in platelets do not show any liver regeneration impairment after PH[259].

Insulin: Insulin is produced by the beta cells of the pancreas, and was one of the earliest identified hepatic mitogens, having been found to prevent liver atrophy when infused directly into the liver *via* the portal stump in dogs having undergone portocaval shunt[11,13]. Although insulin is not a complete mitogen in that it does not induce hepatocyte proliferation *in vitro*, its presence is essential for hepatocyte survival in culture[260] and is essential for the effects of complete mitogens EGF and HGF *in vitro*[261].

The paradox that insulin is not a complete mitogen *in vitro*, but able to prevent liver atrophy after portal diversion is not fully understood but may be partly explained by interactions of the insulin receptor with EGFR and MET, thus triggering those proliferative pathways[212].

Growth hormone and insulin-like growth factor: Growth hormone is synthesized in the pituitary gland and has widespread growth-related roles in many tissues[262]. The effects of GH can be mediated directly *via* the GH receptor, or indirectly by insulin-like growth factor (IGF), which is synthesized by hepatocytes in response to GH and secreted into the circulation bound to IGF binding protein (IGFBP). Whilst hepatocytes do not have IGF receptors[263], Kupffer cells and stellate cells do[264], allowing a possible paracrine role for IGF in the liver. GH may also act directly on hepatocytes by upregulating the EGFR[265] and also stimulating activity of the EGFR in cross-talk with the GHR[266]. Consistent with this, in the rat model, exogenous dietary or infused GH enhances liver regeneration after PH, and mice lacking IGFBP show impaired liver regeneration after PH[63].

In terms of the physiological relative importance of these pathways, Pennisi *et al* [267] showed that GH lacking mice showed the greatest impairment to liver regeneration, with less marked liver regeneration impairment seen in IGF and IGFBP lacking mice, suggesting that whilst both direct and indirect GH actions impact on liver regeneration, the direct effect of GH is more significant that IGF mediated effect.

Thyroid hormone: The thyroid hormones Triiodothyronine (T3) and thyroxine (T4) are produced in the follicular cells of the thyroid gland, and have extensive roles in carbohydrate, protein, and lipid metabolism, regulation of metabolic rate, oxygen consumption, thermal regulation, muscle function, and roles in tissue growth and development[268].

In terms of liver regeneration, thyroid hormones have been shown to act as incomplete mitogens with impaired liver regeneration seen in thyroid receptor knockout mice[269], and conversely accelerated liver regeneration in T3 treated rats after PH[270].

In terms of molecular mechanisms of action in promoting liver regeneration, the thyroid hormones do not act *via* the NFκB or STAT3 pathways which are typically activated by the complete mitogens. Rather, thyroid hormones mediate hepatocyte proliferation by a number of pathways including (1) Increase in expression of transcription factors of the E2F family, which accelerates the transition of hepatocytes from G1 to S phase[271]; (2) Increased expression of cell cycle promotion genes Cyclins A, D1, and E, and diminished expression of their inhibitors[272]; (3) Decreased levels of p53 and p73 (tumour suppressor proteins involved in growth arrest and apoptosis) [269]; and (4) Activation of the *Wnt/b-catenin* signalling pathway[273].

VEGF: The VEGF family of growth factors comprises a group of at least 6 isoforms (VEGF A, B, C, D, E, F), which bind to the 3 different receptors (VEGFR 1, 2, 3), with roles in cell proliferation, migration, metabolism, vasodilation, blood vessel formation and remodelling[274]. Though not directly mitogenic on hepatocytes directly, VEGF plays a central role in liver regeneration in several ways, including the orchestration of proliferation of LSECs, and inducing the LSEC population to produce key hepatocyte mitogens including HGF[175,275].

In a rat 70% PH model, Bockhorn *et al*[276] showed that blocking VEGF signalling with anti VEGF antibodies almost completely suppressed hepatic proliferation in the first 24 h after surgery, and conversely that exogenous VEGF promoted hepatocyte proliferation, suggesting a physiologically relevant role for VEGF in the early stages of liver regeneration.

Complex mitogens

WNT/β-catenin signalling pathway: The WNT family of genes are named after the

gene responsible for the Wingless-type phenotype in *Drosophila melanogaster*, and int-1 (a target for insertional activation of mouse mammary tumour virus, and a secretory glycolipoprotein-encoding gene which is regarded as the prototype for several mammalian genes)[277]. The resulting glycolipoproteins participate in several fundamental signalling events, which influence cell proliferation and tissue homeostasis[278].

β-catenin is a protein encoded by the CTNNB1 and is a subunit of the cadherin protein complex which acts as an intracellular signal transducer in the context of WNT signalling, but which also interacts with a variety of transcription factors such as T-cell factor and hypoxia-inducible factor 1α[279]. β-catenin plays an important role in human embryogenesis, including liver development[280]. It is widely expressed in the adult liver and is always active in the pericentral region. Usually bound to a multiprotein degradation complex, it can be activated by several pathways, including WNT.

The WNT/β-catenin pathway has been shown to be active during liver regeneration, contributing towards mass and functional recovery from a the very early stages of liver injury[281]. β-catenin is in fact detected in rat hepatocyte nuclei within 5 minutes of partial hepatectomy[282]. Upon WNT binding to its receptor (Frizzled), β-catenin translocates to the nucleus, where it promotes the expression of key genes, such as high-level controllers of transcription like *c-myc* and cell cycle regulating genes like cyclin D1[279].

In the normal liver, β-catenin regulates the expression of genes in pericentral hepatocytes and influences hepatic lobular zonation[279] and is involved in cell-cell adhesion[280]. Additionally, it implicated in a variety of diseased liver states, although the exact mechanisms remain incompletely understood. Specifically, β-catenin appears to be involved in the development of NASH, partly by binding to TCF4 and HNF4α, thus regulating hepatic gluconeogenesis and lipogenesis. In hepatic fibrosis, the literature is currently conflicting regarding the role of WNT/β-catenin signalling. Nevertheless, evidence is accumulating to show that this signalling pathway is activating during hepatic stellate cell activation and fibrosis, and that WNT blockade is associated with an antifibrotic effect[279].

This pathway has also been identified in hepatic neoplasia. In focal nodular hyperplasia (FNH), glutathione synthetase (the expression of which is regulated by WNT/β-catenin signalling) stains FNH, which may be of diagnostic value[279]. Other relevant neoplastic processes include hepatocellular adenoma both with and without the presence of CTNNB1 mutations, HCC where mutations may lead to autonomous WNT-mediated activation of β-catenin, and hepatoblastoma, where 90% of tumours are associated with CTNNB1 mutations[279].

Hedgehog signalling pathway: Emerging evidence in the literature has shown the importance of the activation of the Hedgehog (Hh) pathway in the context of liver regeneration. Hh is a protein produced as a 45-kDa precursor that undergoes proteolytic processing in the endoplasmic reticulum[283]. The Hh pathway is a highly complex signalling cascade, which may be summarised in four fundamental components: (1) The ligand Hedgehog; (2) The receptor Patched (Patch); (3) The signal transducer Smoothened (Smo); and (4) The effector transcription factor, Gli. Components of the Hh pathway concentrate in the Primary cilia and a complex Primary cilium trafficking system regulates the interaction of Hh pathway components to enhance, or block, the Hh-initiated signal[284-286].

Previous work in adult rodents has demonstrated that Hh ligand expression increases transiently but significantly following partial hepatectomy[287]. Furthermore, inhibiting Hh pathway induction with a direct pharmacologic antagonist of Smo was found to decrease both recovery of liver volume and overall survival[288]. Evidence in the literature suggests that, mice subjected to portal vein ligation (a procedure commonly done in humans to allow the remnant liver to enlarge prior to hepatectomy) with simultaneous administration of systemic Hh, performed as well as mice submitted to ALPPS, supporting the evidence that Hh signaling plays a major role in promoting liver regeneration[289]. Further evidence suggests that the extracellular matrix of the healthy adult liver, the proteoglycan glypican-3 binds normally to Hh to prevent Hh from binding to Patch in order to constrain activation of the Hh pathway[78].

The evidence above highlights the role of the Hh pathway in post-hepatectomy liver regeneration. Further translational studies are required in order to explore the role of administering a recombinant form of Hh in the pre-operative setting in patients undergoing major hepatectomy who are potentially at risk of liver insufficiency.

Hippo Yap signalling pathway: The Hippo signalling pathway was originally identified in *Drosophila melanogaster* and its components have mammalian homologs [290]. The Hippo signalling pathway exerts a controlling influence on organ size by regulatory effects on cell proliferation, apoptosis, and stem cell self-renewal[291].

The pathway consists of a series of protein kinases, activation of which results in the phosphorylation of yes-associated protein (YAP), thus preventing its translocation to the nucleus. In the nucleus YAP interacts with a family of transcription enhancer factors. This family of nuclear proteins are involved in the modulation and regulation of multiple genes involved in promoting cell proliferation[292-294].

Multiple signals may activate Hippo signalling, including mechanical stimuli and cell attachment. Thus, in situations of high cell density, activation of Hippo signalling leads to the inhibition of YAP nuclear translocation, and thereby a break on cell proliferation[295]. Conversely, decreased Hippo pathway signalling allows YAP mediated pro-proliferation signals and is associated with an increase in organ size through excessive proliferation and inhibition of apoptosis[292]. Consistent with this, and suggesting its physiological importance, mice with liver specific YAP deletions show significant impairment in liver regeneration[296].

However, the Hippo-YAP signalling pathway is the final step of multiple opposing signalling pathways that contribute to liver regeneration and repair and the conflicting nature of these signals makes the study and understanding of the role of this pathway challenging[209].

Intracellular signalling pathways

The complexity inherent in the multitude of extracellular molecules implicated in the control of liver regeneration is reflected by a similarly complex array of intracellular signalling pathways which transmit the effect of ligand-receptor binding to the nucleus to activate effector genes.

In the same way that there is much redundancy in growth factor function extracellularly, there is also much overlap in the intracellular pathways, probably reflecting an evolutionary mechanism to safeguard against failure of any one individual pathway.

The complexity of each pathway, the activation of different pathways by diverse ligands, and the intracellular cross-talk between pathways makes it difficult to assign quantitative importance to any one pathway. Nevertheless, the section describes the main recognized intracellular pathways relating to liver regeneration. A full account of this area is beyond the scope of this review, but the summary below is intended to give a general overview and an impression of the ramifying complexity of the processes involved.

Figure 3A shows the pathways separately in summary form. Figure 3B shows the overlap in ligand binding, and Figure 3C provides an impression of the cross-talk between the various pathways.

Ras/Raf/MEK/ERK pathway: The Ras/Raf/MEK/ERK Pathway is triggered by binding of ligands to receptor tyrosine kinase receptors, which triggers autophosphorylation of tyrosine residues on the intracellular aspect of the receptor, resulting in the sequential activation of downstream components, ultimately controlling the expression of multiple growth controlling genes including high level 'master genes' such as *c-myc, c-fos, and c-jun*[297]. The first molecule, RAS, once activated, can activate multiple different signalling intracellular pathways including not only the Raf/MEK/ERK pathway, but also the MEKK/SEK/JNK pathway, and pathways involving NFκB[298,299].

In liver regeneration, the growth factor which activate this pathway include HGF, the EGFR ligand family members, fibroblast growth factor, and VEGF[299].

Phosphatidylinositol 3'-kinase/AKT kinase (also known as protein kinase B)/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway: The PI3K/ Akt/mTOR is a ubiquitous pathway is involved in the regulation of fundamental physiological processes including transcription, apoptosis, cell cycle progression, and translation [299]. The pathway is activated by binding of ligands to receptor tyrosine kinases or G-protein-coupled receptors[300], ultimately promoting cell growth, proliferation, survival [301], and malignancy when dysregulated[302]. In the context of liver regeneration, the main growth factors activating the PI3K/Akt/mTOR pathway include TNF-α, IL-6, HGF, EGF, and transforming growth factor (TGF)-α[303] (Figure 3B and C).

Janus Kinase pathway: Activation of the Janus Kinase (JAK) kinase pathway promotes cell proliferation, differentiation, migration, growth. Like RTKs, JAKs activate by

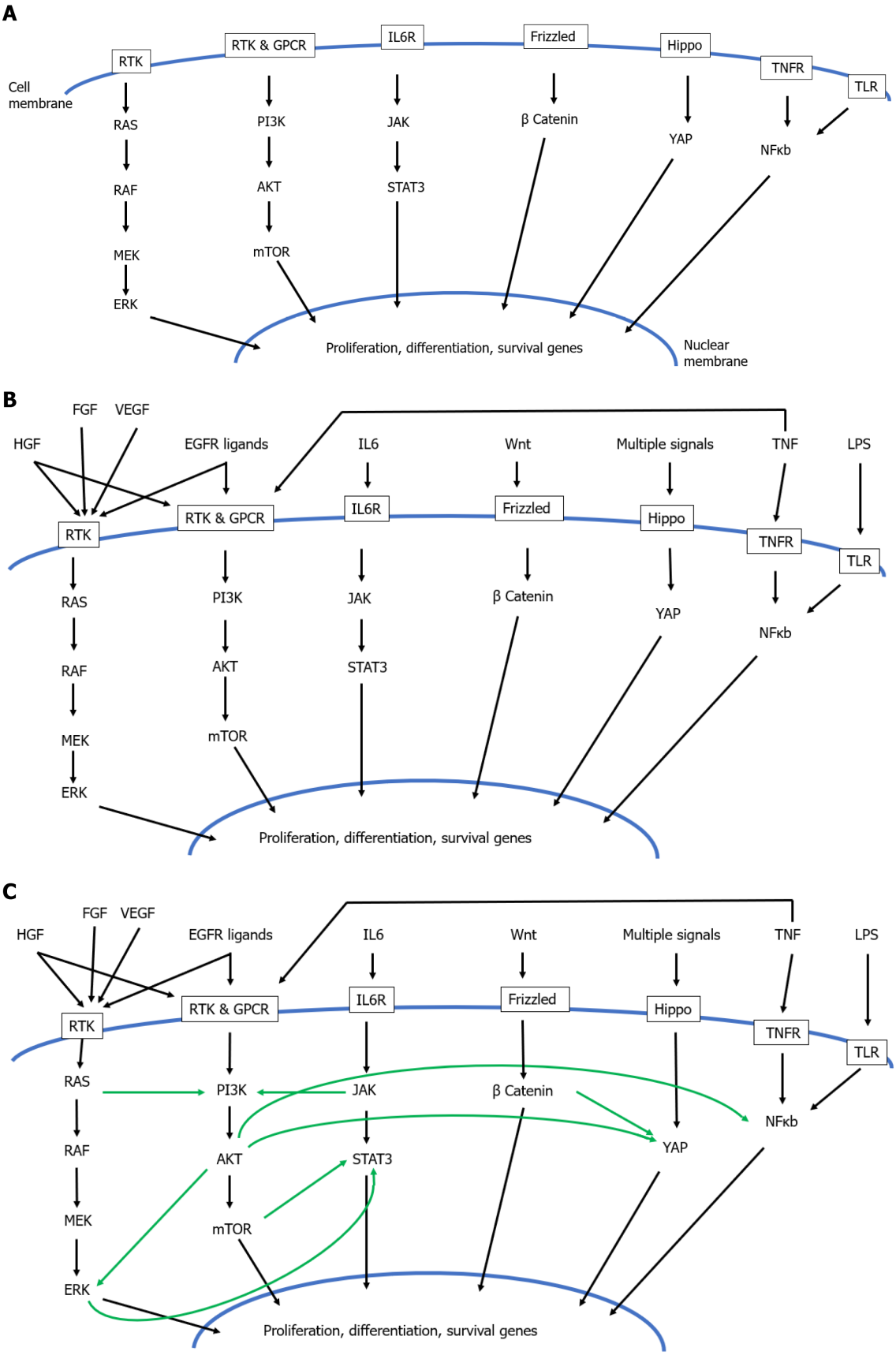


Figure 3 Intracellular signal transduction map. A: Intracellular signal transduction in liver regeneration; B: Ligand overlap and receptor binding redundancy; C: Intracellular cross talk between signalling pathways. HGF: Hepatocyte growth factor; FGF: Fibroblast growth factor; VEGF: Vascular endothelial cell growth factor; EGFR: Epidermal growth factor receptor; IL6: Interleukin 6; TNF: Tumour necrosis factor; LPS: Lipopolysaccharide; RTK: Receptor tyrosine kinase family (including HGF receptor, FGF receptor, VEGF receptor, EGF receptor); GPCR: G protein coupled receptor; IL6R: Interleukin 6 receptor; TNFR: Tumour necrosis factor receptor; TLR: Toll like receptor; RAS/RAF/MEK/ERK: signalling components downstream of receptor tyrosine kinase; PI3K: Phosphatidylinositol 3'-kinase; AKT: Akt kinase

(also known as protein kinase B); mTOR: Mammalian target of rapamycin; JAK: Janus Kinase; STAT3: Signal Transducer And Activator Of Transcription 3; YAP: Yes-associated protein; NFκb: Nuclear factor kappa B.

autophosphorylation. STAT3 is the key downstream messenger which translocates to the nucleus and functions as a high level transcription factor in liver regeneration with resultant induction of gene expression including cytokines[304]. In the context of liver regeneration, this pathway is primarily activated by IL-6 and its receptor, IL-6R.

Interestingly, following PH, a circulating (rather than membrane bound) form of the IL6R cleaved by matrix metalloproteases appears to have a key role in initiating liver regeneration[86].

NF-κB pathway: In priming of hepatocytes, Kupffer cells are induced to secrete TNF-α and IL-6 after stimulation by a variety of stimuli (see section on hepatocyte priming) including complement factors and LPS from the gut. This action of Kupffer cells is mediated by the NF-κB signaling pathway. Once activated, NF-κB migrates to the nucleus, where it promotes the further expression of TNF, IL-6, and VEGF[305].

WNT/β-Catenin pathway: The *Wnt/β-catenin* pathway regulates processes including cell proliferation, and tissue morphology[282]. Upon WNT binding to its receptor (Frizzled), β-catenin translocates to the nucleus, where it promotes the expression of key genes, such as high-level controllers of transcription like *c-myc* and cell cycle regulating genes like cyclin D1[279].

***Hippo* pathway:** The *Hippo* signalling pathway is involved in cell proliferation, apoptosis, and stem cell self-renewal[291], and may have a key role in the ending of hepatocyte proliferation after regeneration[295]. YAP is a key downstream effector of the *Hippo* pathway, which translocates to the nucleus, once activated to promotes expression of target genes. The pathway is activated by numerous factors including organ size, cell attachment, mechanical stress, hormones, growth factors[295], as well as vascular shear stress[306].

Cross-talk between pathways: Mirroring the redundancy and overlap in extracellular growth factors and receptor binding, there exists a complex crosstalk between pathways. Just a few examples of the known positive feedback interactions are shown in Figure 3C, which, albeit incomplete and without including negative feedback interactions, provides an impression of the intricacy of intracellular interactions between pathways[297]. Thus, Ras interacts with phosphatidylinositol 3′-kinase (PI3K)[307]. The NF-κB pathway cross-talks with the PI3K/Akt/mTOR, pathways[308]. β-catenin interacts with the Hippo signalling pathways[195]. YAP cross-talks with PI3K/Akt pathway[309].

SECTION 4: THE CONTRIBUTION OF NON-PARENCHYMAL CELLS TO LIVER REGENERATION

The proliferation of hepatocytes in liver regeneration is in critical ways dependant on the role of non-parenchymal cells (Kupffer cells, stellate cells, and liver sinusoidal endothelial cells). Conversely, proliferating hepatocytes provide many growth factors that elicit non parenchymal cell proliferation, thus suggesting an interdependence allowing proportionate expansion of each cell type to produce liver tissue containing all key constituents. Thus, proliferating hepatocytes produceVEGF and angiopoietins 1 and 2 (LSECs mitogen), TGFα (LSECs and stellate cell mitogen), fibroblast growth factor 1 (FGF1) and FGF2 (HSC and LSEC mitogen), and granulocyte–macrophage colonystimulating factor (GMCSF) (Kupffer cells mitogen)[191,310,311]. In this section we examine in more detail the part played by non-parenchymal cells in liver regeneration.

Liver sinusoidal endothelial cells

LSECs have a key role in the immediate events that trigger the onset of hepatocyte proliferation after PH. Thus, shear stress resulting from portal pressure changes after PH induces the expression of the hepatocyte priming IL6[164].

Once liver regeneration is initiated, hepatocytes form clusters which are initially avascular. The production of VEGF, and angiopoietin 1 & 2 stimulates endothelial cells to migrate into the avascular structures, proliferate, differentiate to a liver sinusoidal phenotype[312]. LSECs also produce VEGF[313], as well as the potent mitogen HGF, and NO[160,162] which increases hepatocyte sensitivity to HGF[163].

In the rat hepatectomy model, liver sinusoidal cell repopulation is not only achieved by resident LSECs, but also by recruitment of LSEC progenitors in the bone marrow which, under the influence of VEGF, undergo proliferation, migration into the bloodstream, and engraftment in the liver, where they contribute a significant proportion of the total HGF production.

Of note, the importance of bone marrow derived LSECs in this model is emphasised by the fact that bone marrow ablation by irradiation abolishes liver regeneration, which can be rescued by exogenous infusion of LSECs[314,315]. Thus, LSECs play a key role, not only in allowing the vasculature of the liver to keep pace with regenerating hepatocytes, but also in providing the very growth factors (such as HGF) that allow hepatocyte to proliferate.

Stellate cells

Stellate cells are situated in the Space of Disse between LSECs and hepatocytes [316], and though representing only approximately 8% of cells in the liver, have multiple long cytoplasmic projections[317] which contact hepatocytes, LSECs, and Kupffer cells, allowing a central role in intercellular signalling in part by production of growth factors such as HGF and VEGF. Stellate cells also exhibit cellular contraction, permitting the control of sinusoidal blood flow[318], and have a key role in the regulation of ECM, both in its production and degradation[319].

After hepatic injury, HSC become activated myofibroblast-like cells. In the midst of liver regeneration, the initiation, perpetuation and resolution of HSC activation only adds further complexity, in processes which are poorly understood[320], but which ultimately result in the laying down of ECM to provide the vital framework on which regeneration may proceed[321].

The triggering of HSC activation is multifactorial, but includes the secretion by hepatocytes of growth factors such as FGF1 & 2, and PDGF, the latter being a potent mitogen and chemo-attractant for HSCs[322], and highlighting an example of interdependent complex paracrine stimulation (with HSCs producing the hepatocyte mitogen HGF and hepatocytes producing the HSC mitogen PDGF).

Following activation, HSC production of HGF increases, in a mechanism dependant on the neurotrophin receptor P75NTR[323], and its downstream mediator Rho[324]. Activated HSCs also produce Noradrenaline, which enhances HGF production by mesenchymal cells[223] and production of EGF from Brunner's glands[229]. HSCs interact directly with LSECs to stabilise and remodel sinusoids[325], *via* combined actions of PDGF, TGF-β1, FGF, VEGF, and angiopoietin. HSC are the principal cell source of ECM constituent production, and of ECM remodelling control by expression of matrix metalloproteases, thus providing the scaffold in which liver cells can regenerate[326].

The importance of HSC activation in liver regeneration is suggested by the observation that following ablation of HCS activation, liver regeneration is markedly impaired in both the mouse acetaminophen[327] and rat acetyl amino fluorene[328] models of liver injury with much reduced proliferation of hepatocytes and oval cells respectively, and with rescue of liver regeneration by infusion of medium conditioned by HSC[329].

Kupffer cells

Amongst the different resident intrahepatic macrophages, Kupffer cells are the predominant type, and originate from erythromyeloid progenitors in the foetal liver [330]. In the homeostatic situation, Kupffer cells have wide-ranging roles in (1) Clearance of cellular debris in blood[331]; (2) Maintenance of iron homeostasis *via* phagocytosis of red blood cells[332]; (3) Regulation of cholesterol homeostasis[333]; (4) Antimicrobial defence[334]; and (5) Promotion of immunological tolerance[335].

Kupffer cells have a limited half-life (of approximately 12 d in mice)[336]. Their maintenance is achieved by self-replenishment in the healthy liver[337], but is to an extent dependant on extra-hepatic progenitors in the case of liver injury[338].

There is evidence that replenishment of Kupffer cells following injury may be achieved by engraftment and differentiation of monocyte derived macrophages into a Kupffer cell phenotype, in a manner controlled by HCS and LSEC, highlighting once again the complex cross-talk between the non-parenchymal cell types[339]. The monocyte derived macrophage precursors may originate from within the liver, but

also from the peritoneum[340] and spleen[341].

Kupffer cells are strategically placed with access to the sinusoidal lumen and space of Disse, and carry multiple surface receptors to injury related molecules (the Pattern Recognition Receptors), suggesting a key role for Kupffer cells as sensors of hepatic injury[342].

Thus, Kupffer cells are able to detect, and become activated by: (1) DAMPs, *e.g.*, mitochondrial DNA (mtDNA), and ATP, from damaged hepatocytes; (2) Pathogen-associated molecular patterns (PAMPs)[343]; (3) Hypoxic liver environment[344]; and (4) Extracellular vesicles secreted from various cells containing proinflammatory stimuli[345].

The activation of Kupffer cells by any of the above stimuli results in the secretion a wide range of bioactive molecules including chemokines such as C chemokine 2 which attract inflammatory and immune response cells to the injury site[346], and proinflammatory cytokines such as IL6 and TNFα which prime hepatocytes out of G0 phase an also activate HSCs[347].

Thus, Kupffer cells have a central role in the initiation and orchestration of liver regeneration, and their importance is suggested by the observation that depletion of macrophages[206] or inhibition of monocyte[348] recruitment results in impaired liver regeneration following PH.

SECTION 5: THE 'ALTERNATIVE PATHWAYS' OF LIVER REGENERATION

The liver regenerative response varies not only according to the nature of the injury, but also its magnitude and the status of the underlying liver parenchyma. These different contexts dictate how regenerating cells behave, but also the recruitment of different types of cells to accomplish the task. Current knowledge on the mechanisms of liver regeneration is largely derived from experimental models involving 2/3 PH in rodents[195], where "standard" liver regeneration occurs. This involves the proliferation of hepatocytes and cholangiocytes from homotypic precursors[209] and is addressed in the first subsection below. In the second subsection, the "alternative" liver regeneration pathways, which involve liver progenitor cells (LPCs) and transdifferentiation, will be examined. As seen in other aspects of liver regeneration, the mechanisms outlined below are subject to ongoing scientific scrutiny and are currently incompletely understood.

Hepatocyte response heterogeneity after PH

Liver regeneration after PH is achieved in different ways according to the magnitude of the liver resection. Thus regeneration after 1/3 PH is achieved principally by hypertrophy, with few cell divisions[349]. In contrast, during liver regeneration after resections larger than 1/3PH, although hypertrophy precedes hyperplasia, hyperplasia occurs increasingly as well, such that hypertrophy and hyperplasia contribute equally to liver regeneration in 70% PH. Moreover, during the hyperplastic response, although the majority of hepatocytes entered S phase of the cell cycle, not all undergo actual cell division, and the known significant number of polyploid hepatocytes[350] are shown to undergo division to produce mononuclear hepatocytes.

Thus, hepatocyte behaviour during liver regeneration is not uniform. Hepatocytes within a liver lobule are not equivalent and show functional heterogeneity. The liver lobule may be separated into 3 zones: Zone 1 (periportal) hepatocytes are in the vicinity of the portal triad, zone 3 (perivenular) hepatocytes are situated near the central vein, and zone 2 (pericentral) hepatocytes reside between zones 1 and 2. Metabolically speaking, zone 1 hepatocytes carry out gluconeogenesis and b-oxidation, in contrast to glycolysis, lipogenesis, and detoxification performed by zone 3 hepatocytes[195].

Moreover, there is some evidence that there is heterogeneity in baseline hepatocyte turnover during homeostasis, with a population of cells in zone 3 replenishing the lobule population, albeit slowly[351].

Hepatocyte proliferation heterogeneity is also apparent in the context of the regenerative response. In the context of true proliferative response after PH, lineage experiments have identified a population of hepatocytes in the periportal region zone 1 which appear to have greater proliferative potential. These are referred to as 'hybrid hepatocytes 'in that in addition to hepatocyte markers, they express progenitor cell genes and biliary transcription factors[352]. There appears to be further heterogeneity in proliferative response in that hepatocytes in proximity to LSEC proliferate faster than ones which are more distant[175].

Facultative stem cells and transdifferentiation pathways

The alternative liver regenerative pathways are characterized by deviation from the phenotypic fidelity in which hepatocytes or cholangiocytes proliferate to produce more of the same cell type. In this "alternative" context, liver epithelial cells (*i.e.*, hepatocytes and cholangiocytes) can operate as facultative stem cells for one another in conditions where regeneration of one or other is impaired[209,353], presumably as a rescue mechanism. This mechanism appears beneficial from an evolutionary standpoint, is plausible from a developmental biology perspective given that both hepatocytes and cholangiocytes are derived from hepatoblasts[354], and has been demonstrated in previous studies[81,355-357]. Nevertheless, there is controversy in the field regarding the *in vivo* capability, conditions, and extent to which liver epithelial cells can transdifferentiate and achieve regeneration.

The term "LPCs" is seen in the literature, yet such cells have not been identified on microscopy or tissue dissociation of liver lobules in the resting state and are thought to possibly arise from the transdifferentiation of hepatocytes and cholangiocytes[353] as mentioned above. A central event in this process is the "ductular reaction", which occurs when hepatocyte proliferation is suppressed, thus leading to the expansion of progenitor cells[209] and which can be observed both in acute and chronic liver disease models, typically after extensive hepatocyte injury.

In conditions such as fulminant hepatic failure, some liver epithelial cells demonstrate an overlapping set of biomarkers (*e.g.*, cholangiocytes may express "hepatocytic" biomarkers such as HNF4, albumin, and HEPPAR3)[353]. The extensive necrosis and apoptosis characteristic of fulminant hepatic failure is thought to pivot liver regeneration towards LPCs, as reflected in elevated α-fetoprotein levels[358] and as demonstrated by histological findings of "regenerative clusters", which consist of atypical ductules lined by cells exhibiting a combination of cholangiocyte and hepatocyte biomarkers[209].

In the context of PH, once a certain resection threshold is exceeded (*e.g.*, > 80%), adequate liver regeneration cannot be achieved by the relatively small number of remaining hepatocytes, and the alternative pathway is thus activated. In this process, biliary epithelial cells (cholangiocytes) de-differentiate into progenitor cells and then re-differentiate into hepatocytes in order to repopulate the liver[195]. As described in the rat models subsection above, Evarts *et al*[359] demonstrated that administration of 2-AAF to rats which had undergone PH was associated with differentiation of oval cells (a putative hepatic progenitor cell) to hepatocytes. However, this model did not allow for genomic-based cell lineage tagging[209].

More recently, Lu *et al*[81] induced widespread hepatocyte injury in mice through Mdm 2 deletion, which results in p53 upregulation with p53-induced hepatocyte death and senescence. The authors found that widespread hepatocyte injury was associated with a ductular reaction, whereby hepatocyte progenitor cell populations expanded and where bromodeoxyuridine-positive hepatocyte progenitor cells were often closely associated with bromodeoxyuridine-positive hepatocytes, thus suggesting that (in this context) hepatocytes arise from progenitor cells[81]. In a zebrafish model, Choi *et al*[355] found that after severe hepatocyte depletion, biliary epithelial cells de-differentiated into hepatoblast-like cells and then differentiated into highly proliferative hepatocytes, thus leading to liver regeneration.

Although the above studies focused on transdifferentiation from cholangiocytes to hepatocytes, the inverse has also been demonstrated in a murine model of Alagille syndrome (a human genetic condition associated with biliary underdevelopment). In this study, Schaub *et al*[360] found that hepatocytes converted to mature cholangiocytes that were effective in supporting biliary drainage and remained so after cholestasis resolved, in a TGFβ signalling mediated process. This persistent phenotypic change is distinct from the reversible conversion of human or murine hepatocytes to progenitor cells seen in other studies[361] and which may more accurately be described as "metaplasia" rather than "transdifferentiation". In their chimeric liver rat model, Michalopoulos *et al*[362] injected dipeptidyl peptidase IV (DPPIV)-positive rat hepatocytes into DPPIV-negative rats which then underwent partial hepatectomy and bile duct ligation, with or without additional biliary injury by methylene diamiline (DAPM) administration. On animal sacrifice after 30 d, the authors found that ductules exhibited the DPPIV marker, and that this was enhanced 36-fold in rats with additional DAPM-mediated biliary toxicity.

Evidence in support of liver epithelial cell transdifferentiation for regeneration is accumulating, yet several areas of controversy remain to be resolved. In addition to the findings described above, self-renewing facultative stem cells have been located in peribiliary glands and liver progenitor cells of bipotential differentiation capacity have been located in association with the canals of Hering. However, their role in liver

regeneration has been disputed[195]. Also, it remains unclear whether all mature hepatocytes are capable of dedifferentiation to progenitor cells, or whether this is only possible in a subset of cells[195]. Finally, various signalling pathways (*e.g.*, YAP, Rho kinase, TGF-β, glycogen synthase kinase 3) have been implicated in hepatocyte dedifferentiation in animal models[195], yet their role with respect to human hepatocytes remains to be clarified.

SECTION 6: THE INFLUENCE OF UNDERLYING LIVER DISEASE ON LIVER REGENERATION

The processes and mechanisms of liver regeneration are not only influenced by the magnitude and type of injury, but also by the status of the underlying liver parenchyma prior to injury. Although the PH model has contributed much information relating to events relating to regeneration of normal liver, significant differences come to light when the underlying liver is diseased. This section describes the ways in which liver regeneration is altered in the instances of age-related liver impairment, acute liver injury, hepatic steatosis, fibrosis, and cirrhosis.

Age-related liver impairment

Although the adult liver retains regenerative capacity throughout life, this is reduced in old age[363], through several suggested mechanisms. FoxM1B is a transcription factor expressed during embryonal development and also in liver regeneration[364]. Its expression is diminished in aged mice, whose liver regeneration can be rescued through its transgenic overexpression[365]. Age-related liver regeneration impairment may also be mediated by changes in the expression and function of cell cycle affecting genes such as CCAAT/enhancer-binding protein (C/EBP)α, which is an inhibitor of Cyclin D[366]. Budding uninhibited by benzimidazole-related 1 (BubR1) is involved in the control of mitosis and is found to be diminished in old age. Genetically manipulated mice expressing low levels of BubR1 show impaired liver regeneration [367].

In addition to these mechanisms, Conboy *et al*[368] and Liu *et al*[369] have demonstrated in parabiotic experiments that the blood or plasma of young mice partially rescues the liver regenerative compromise seen in old mice, suggesting the presence of currently unidentified circulating factors, in work reminiscent of the early experimental approaches used to demonstrate the existence of portal mitogens.

Acute liver injury

Severe acute liver injury may result from a variety of insults including viral infection (*e.g.*, Hepatitis A, B, C), poisoning (*e.g.*, paracetamol) or auto-immune disease. Though different in nature, these diverse insults nevertheless have the common feature of causing significant necrosis and apoptosis, in the midst of which regeneration must happen.

Specific models provide some mechanistic information. For example, the mouse model of paracetamol injury suggests that beyond a threshold of injury, regeneration fails, and that this is associated with failure of β catenin activation, consistent with the correlation of β-catenin activation and regeneration seen in patients[370].

In the setting of widespread necrosis, the contribution of non-parenchymal cells may be particularly important. Macrophages are essential in clearing toxic cellular debris[371], and thus mice deficient in CSF1 which promotes the maturation of macrophages have impaired liver regeneration[372], and CSF1 serum levels correlate with recovery from paracetamol liver injury[373].

Hepatic steatosis

Hepatic steatosis is known to be detrimental to liver regeneration not only in experimental models[374], but also in the clinical setting[375]. The mechanism is not fully understood but may include cell cycle machinery defects in steatosis[376]. Down regulation of the EGFR pathway may also contribute, and EGFR overexpression has been shown to rescue liver regeneration in a mouse model of PH in steatosis[377]. Steatosis may also compromise liver regeneration by inhibition of NFκB[378]. Finally, failure to activate growth arrest and DNA damage-inducible protein GADD34 in fatty liver may partly contribute to impaired liver regeneration, which can be rescued by transgenic overexpression of growth arrest and DNA damage-inducible protein GADD34 in a mouse experimental model[379].

Hepatic fibrosis and cirrhosis

In the setting of overwhelming injury where the capacity of hepatocytes to proliferate is overwhelmed, oval cell transdifferentiation provides a mechanism to assist cellular repopulation. Such injury is, however, also associated with activation of stellate cells to myofibroblasts, which secrete ECM[380]. Excessive ECM secretion is nonetheless harmful because it inhibits the ductular reaction[381] and hepatocyte proliferation [382]. Moreover, excessive fibrosis impairs portal flow, leading to arterialisation of the liver, and senescence of hepatocytes and cholangiocytes[383]. The distortion of the macro and micro-anatomy of the liver results in major compromise to liver function leaving affected patients in an extremely precarious state characterized by rapid and severe decompensation, which can be triggered by relatively minor physiologically stresses.

SECTION 7: MECHANISMS UNDERLYING CESSATION OF LIVER REGENERATION

In the rodent PH model, liver regeneration proceeds until the liver have returned to its pre-PH weight, approximately 10 d later, at which point regeneration ceases. The mechanisms resulting in termination of liver regeneration have received less attention than those driving it, but are equally important, not only from the perspective of the study of liver regeneration biology but also in terms of the light they shed on other pathologies including liver malignancy. Nevertheless, several regeneration termination pathways have been identified, relating to TGFβ, the activins, the ECM, and glypican-3 (GPC3).

TGF β

TGFβ is a multifunction cytokine with wide ranging roles in growth and development. It exists in 3 isoforms resulting from differential protein cleaving and binds to 3 different TGFβ receptors. Binding to the TGFβR results in autophosphorylation, and activation of SMAD, which translocates to the nucleus, delivering an inhibitory signal to cell proliferation[384]. Although TGFβ does inhibits hepatocyte proliferation *in vitro* [385], other experimental results *in vivo* cast some doubt on its role in termination of liver regeneration in that liver specific TGFβR knockout mice terminate liver regeneration appropriately[68]. However, this result does not necessarily rule out TGF β as a significant factor in liver regeneration termination: given the redundancy seen in the processes that drive regeneration, it seems likely that similar redundancy exists in its termination, such that ablation of one mechanism may readily be rescued by other pathways.

Activins

The activins are a family of proteins which are similar in structure to the TGFβ family, which also transduce signals *via* receptors that activate SMAD, and convey a growth inhibitory effect. Activins are upregulated during the liver regeneration[386], and blocking their action pharmacologically results in excessive regeneration and hepatomegaly following PH in rats[387].

ECM and integrin linked kinase

The ECM is thought to convey a growth controlling influence on liver cells in a mechanism whereby integrin proteins in intact ECM bind hepatocyte cell membrane Integrin linked kinase (ILK) receptors, which deliver a growth inhibitory signal[388]. Consistent with this, the growth response of hepatocytes to mitogens *in vitro* is much reduced when grown in the presence of ECM in comparison to plastic[389]. Moreover, ILK knockout mice show not only hepatomegaly in the native state[390], but also an exaggerated regeneration after PH[391]. Thus, it may be that the activation of matrix metalloproteases that occurs early after liver injury[188] results in degradation of the controlling influence of integrins, and that this is gradually recovered during regeneration as new ECM is laid down.

GPC3

GPC3 is a heparan sulphate proteoglycan found on the cell surface of many tissues which conveys a growth inhibitory effect[392]. It is not detectable in quiescent liver but is expressed coinciding with the end of regeneration after PH in rats[393]. Moreover, loss of function mutations of GPC3 results in organ overgrowth[394], and transgenic

over-expression of GPC3 delays liver regeneration after PH[393].

C/EBP

C/EBP is one member of a family of transcription factors with a role in producing cell cycle arrest. Although complete ablation of C/EBP is fatal in mice[395], altering the function of the protein by mutation results in partial loss of function with mice exhibiting excessive regeneration and hepatomegaly after PH and CCl4 injury[396], thus suggesting that the native protein has a role in the control of liver regeneration.

Cyclin E1 and E2

The cyclins are a group of proteins which impact on the progression of the cell through the cell cycle. Amongst these cyclins E1 and E2 influence the advancement of the cell from the G1 phase (during which the cell synthesizes protein and organelles), to S phase (during which DNA is replicated)[397]. Cyclin E1 and E2 have opposing roles, with Cyclin E1 promoting entry into S phase[398], and Cyclin E2 halting it[399]. Thus, mice with ablated Cyclin E2 show increased DNA synthesis and hepatomegaly after PH suggesting a role for Cyclin E2[398,400].

Of note the hepatomegaly seen after CyclinE2 ablation is not due to cell division, but hypertrophy, providing a possible mechanism to explain to the observation that liver growth after 30% PH is hypertrophic rather than the hyperplasia seen in 70% PH.

Hippo/YAP pathway

The *Hippo/Yap* signalling pathway is conserved in a wide range of organisms and associated with growth suppression[401]. *YAP* is key downstream effector of *Hippo* and its activation when dephosphorylated leads to massive liver overgrowth[402], suggesting a possible regulatory role in control of liver regeneration.

Micro RNAs

Micro RNAs are short RNA molecules which bind to messenger RNA and thus affect expression of the gene product by interfering with translation of mRNA to protein [403]. Several micro RNAs have been identified which target the mRNAs of key regeneration promoting proteins, and thus may play a part in controlling liver regeneration. Thus miR-23b targets the growth inhibiting SMAD protein such that the observed downregulation of miR-23b following PH may provide a mechanism for slowing liver regeneration[404]. miR-34a targets several mRNAs including that which codes for the HGF receptor MET, again providing a potential mechanism for limiting hepatocyte proliferation[405].

SECTION 8: LIVER REGENERATION: IMPLICATIONS FOR THERAPY OF LIVER TUMOURS

The complexity of liver regeneration biology, combined with our currently limited understanding has to date much restricted specific clinical interventions to enhance liver regeneration. Moreover, the processes involve such fundamental biochemical pathways that attempts to manipulate these would require very careful assessment, for fear of unintended consequences in the liver and other organ systems. Nevertheless, current knowledge allows clinicians to anticipate what scenarios or treatments may compromise liver regeneration and provides guiding principles which may allow planning treatment strategies to optimise liver regeneration potential.

Liver tumours, be they primary or metastatic, may be treated by chemotherapy in systemic or locoregional delivery methods, or by surgical intervention with resection or local ablation techniques. Radiotherapy, although used to an extent, has a much lesser role and evidence base. In this section, we discuss how the biology of liver regeneration affects treatment choices and delivery, not only in terms of chemotherapy and surgery, but also in relation to wider organ system physiology that relates to liver regeneration.

Chemotherapy and liver regeneration

The principal scenario in which chemotherapy impacts on liver regeneration is the use of neoadjuvant chemotherapy prior to a planned liver resection, or in the instance of downsizing an initially unresectable lesion, most usually in the context of colorectal liver metastases[406]. Chemotherapy affects not only individual cell types within liver parenchyma, but also key extra-hepatic tissues pertinent to the liver regenerative

process such as the bone marrow, and in some cases affects key liver regenerative pathways.

The main toxicities inflicted on the liver by chemotherapy are steatosis, steatohepatitis, and sinusoidal obstruction syndrome (SOS). Steatosis is the excessive deposition of fat within hepatocytes. This may trigger an inflammatory reaction leading to steatohepatitis, and in turn to fibrosis and cirrhosis. Sinusoidal obstruction syndrome is a separate entity with direct toxicity to LSEC leading to occlusive phenomena in the sinusoids[407].

In the context of treatment of (colorectal liver metastases) CRLM, 5FU is associated with steatosis[408], probably as a result of impaired oxidation of fatty acids[409]. 5FU also triggers the activation of pro-inflammatory genes, which may contribute to the evolution of steatohepatitis[410]. Chemotherapy associated Steatohepatitis (CASH) is also particularly associated with regimens containing irinotecan[411] which also inhibits fatty acid oxidation, but elicits steatohepatitis by activation of ERK[412], which if inhibited, leads to a reduction in steatohepatitis. SOS is associated with oxaliplatin regimens. Although not fully understood, a contributing mechanism is the increased expression of matrix metalloproteases, resulting in lifting of LSEC from the basement membrane and allowing infiltration of red blood cells into the space of Disse, thus causing an occlusive phenomenon in the lumen of the sinusoid, and an inflammatory reaction with stellate cell activation and perisinusoidal fibrosis[413].

In addition to these directly hepatotoxic effects, the above agents are myelosuppressive, and may thus compromise the bone marrow constituents that play important roles in liver regeneration, including (1) LSEC progenitors which are important in repopulating LSEC after PH and a key source of the important mitogen HGF[314,315]; (2) Macrophage progenitors which have a key role in clearing cell debris in preparation for regeneration[206]; and (3) Megakaryocytes which replenish platelets, with their important role in delivering liver regenerative signals[178,179].

5FU, oxaliplatin, and irinotecan are frequently used in combination with biological agents targeting specific proliferative pathways within tumours, which overlap with biochemical pathways promoting liver regeneration. Thus, the anti-EGFR antibody cetuximab blocks binding of EGFR ligands including the key mitogen EGF, acting *via* the RAS-RAF-MEK pathway. Another example is the anti-angiogenic antibody bevacizumab which delivers antitumor effects by blocking VEGF-binding to its receptor, but thereby also interfering with LSEC repopulation in the liver[406]. Thus, the commonly used chemotherapeutic agents and antibodies used in the treatment of CRLM have a multitude of liver regeneration compromising properties. The challenge for clinicians is to find the optimal balance between the oncological benefits and liver regenerative toxicity.

Clinical trials provide some guidance in this regard. Thus 5FU, oxaliplatin and irinotecan-based regimens have been shown to produce CRLM response rates allowing increased potential for curative resection[414,415], with additional benefits attributed to the use of anti EGFR[416] and anti VEGF antibodies[417]. Interestingly, whilst the use of EGFR antibody regimens is established as a means to downsize tumours to increase operability, their perioperative use in primarily resectable CRLM is detrimental[418].

In terms of duration of chemotherapy, the theoretical ideal is the delivery of the oncological hit to the tumour whilst minimising liver toxicity. In this regard Karoui *et al*[419] found that patient receiving fewer than 6 chemotherapy cycles experienced significantly fewer post liver resection complications than those who had received more than 6 cycles (19% *vs* 54% complication rate) although there was no impact on mortality rates. Similarly, Nguyen *et al* [420]showed a greater incidence of post-operative liver failure in patients undergoing more than 10 cycles of chemotherapy. Moreover, the general practice of leaving an interval of 4-6 wk between chemotherapy and surgery is intended to allow reversible inflammatory changes and bone marrow to recover[421].

The prospect of specific interventions to minimise chemotherapy related injury is the subject of research but has not yet reached widespread clinical application. Nevertheless, there have been reports that bevacizumab may reduce SOS in oxaliplatin regimens[422], and S-adenosylmethionine (SAMe) may have a protective effect in chemotherapy-induced liver injury[423], with SAMe infusion associated with lower serum concentrations of aspartate transaminase and alanine transaminase during chemotherapy treatment[424].

Liver volume manipulation

In the context of a healthy underlying liver parenchyma, up to 75% of the liver may be resected. However, this is the very limit and liver surgeons are often faced with the

problem of some degree of parenchymal pathology or dysfunction, which may present a higher requirement in terms of the remnant liver volume. As discussed in section 1.4, pure volume assessments are gradually being complemented by liver functional assessments, principally by hepatobiliary scintigraphy[146,152].

In instances where future remnant liver volume/function is deemed insufficient, PVE has become an established technique to produce atrophy of the tumour bearing liver parenchyma, and compensatory growth of the future remnant liver, allowing a safer hepatectomy[425]. In the context of resections for CRLM, this technique increases resection rates by 10%-20%[426].

In the instance where PVE fails to produce sufficient hypertrophy, perhaps caused by the development of collateral intrahepatic portal vessels between the embolized and non-embolized parts of the liver, further growth of the future remnant liver may be achieved by parenchymal section to interrupt the collateral vessels.

This approach, as a salvage manoeuvre after PVE is a modification of the originally described ALPPS technique[427] that combined single stage portal vein embolization and parenchymal transection. Whilst undoubtedly producing significant additional growth, the technique remains debated owing to questions regarding the functionality of the rapidly expanded liver, and high post-operative mortality in some series[428].

Liver disease limitations to liver resection

The continuum of steatosis, steatohepatitis, fibrosis, and cirrhosis presents a major challenge to liver resection. In terms of steatosis, the observed experimental and clinical compromise to liver regeneration as a result of steatosis translates to a diminished tolerance to liver resection[429]. Thus, studies examining the outcome of hepatic resection in patients with steatotic livers suggest more marked abnormalities in postoperative liver dysfunction, more morbidity and increased complication rates [143,430], with steatosis identified as an independent predictor of complications[431]. Increased mortality is identified in some studies[430], and although this has not been a universal finding, the presumption is that this relates to careful patient selection.

The tolerance of the liver to resection progressively decreases with more severe underlying liver disease. Thus, cirrhosis is associated with increased mortality rates for all abdominal surgery including[432] liver resection[433].

Interventions to mitigate the risk of liver surgery in the context of underlying liver disease are limited, and practice has focused on patient selection to avoid prohibitively hazardous resections. In the context of steatosis, the response of the steatotic liver to ischaemic insult[434] has motivated research in the concept of ischaemic preconditioning, whereby a short period of ischaemia prior to liver resection is applied with a view to improving subsequent perfusion[435]. Although benefits have been shown in rodent models and in the clinical setting[436], results have not been universal[437], and the practice not widely accepted.

The role of the gut

One of the key biochemical triggers which initiates liver regeneration is LPS from the gut, which translocates into portal blood following liver resection in part as a result of the rise in portal pressure[204] (see "*Priming of hepatocytes*"). The evidence supporting the importance of LPS is that germ free mice show impaired liver regeneration, which can be rescued by exogenous LPS administration[205].

Though the presence of LPS in the blood is important, there appears to be a delicate balance as excessive translocation of LPS into portal blood is detrimental to liver regeneration. Thus, in the rat model after 90% PH, gut mucosal permeability is disrupted with loss of tight junctions, resulting in high levels of portal blood LPS, severe inflammatory changes in the liver with necrosis, associated with high mortality. By decontaminating the gut with gentamycin, gut permeability, portal blood LPS, and liver necrosis is much improved, and associated with a significant improvement in survival from 24% to 56%[27].

No clinical trials have been carried out to test the potential benefit of this animal experimental result in humans, where its applicability could be investigated in patients undergoing major liver resection. Additional considerations would come into play, including the risk of *clostridium difficile* sepsis associated with alteration of bowel flora. Another potential approach, also uninvestigated, could be the use of bowel preparation similar to that used prior to colorectal surgery.

Multivisceral resections

The data from early experimental result suggested that liver regeneration was significantly dependent on portal blood growth factors derived from the upper

gastrointestinal tract (stomach, duodenum, pancreas) as portal flow separation experiments comparing isolated portal flow of distal stomach, duodenum, pancreas and spleen to portal flow of small intestine showed that the grafts supplied with intestinal portal flow atrophied, in contrast to those supplied with portal blood from the upper gastrointestinal tract[9,10].

On this basis, there might be a similar risk in humans in cases where liver resection is combined with a significant resection of the upper gastro-intestinal tract, such as Whipples resection (resection of distal stomach, duodenum and head of pancreas) or total pancreatectomy (resection of distal stomach, duodenum, pancreas, and spleen). However, existing case series describing such multivisceral resections do not report problems relating to liver regeneration[438-440].

Thus it may be that the situation in the original animal experiments cannot be extrapolated to humans owing to different physiology, although interpretation of the human literature has to be taken with the caveats of selective reporting (bias against reporting poor outcomes with liver failure), case selection of surgical candidates (likely good performance status individuals for such major resections), and likely small liver resections which might not manifest with post-operative liver failure.

Bile metabolism considerations

Bile salts are important auxiliary mitogens, with rodent models showing impaired liver regeneration with bile acid sequestering agents[243], bile-salt-deficient transgenic mice[244], and in rats with external biliary fistula[245].

This observation in rodent models is mirrored in clinical practice, with a randomised trial comparing patients undergoing major liver resection with and without cystic duct biliary drainage showing significantly lower bile salt concentrations in the drained group as well as lesser liver regeneration assessed by volumetric CT[441]. Moreover, in the context of portal vein embolisation, increased systemic bile salt levels predicted hypertrophy of the non-embolised lobe[442].

Thus, the presence of bile in the intestine following liver resection appears to be important and would argue against the use of external biliary drains after liver resection, or in circumstances requiring such drains, to consider enteric bile recycling *via* nasogastric or nasoenteric tube.

Management of post-hepatectomy portal hypertension

The rise in portal venous pressure following liver resection appears to contribute to providing important triggers to liver regeneration in the form of induction of cytokines, hepatic mitogens and angiogenic growth factors, as described in "*Vascular events*"section. However, an excessive increase in portal pressure is thought to be detrimental in eliciting a reduced arterial inflow as a result of the arterial buffer response, and a subsequent hypoxia[171], which is hyptothesised to contribute to post liver resection liver failure[443], in combination with direct mechanical injury occurs to the liver sinusoids[444].

Thus, a number of investigators have examined a variety of interventions to decrease portal venous pressure by surgical (splenectomy, splenic artery ligation, porto-systemic shunt), interventional radiological (pre-operative splenic artery ligation), and pharmacological means (non-selective beta-blockers, terlipressin), with successful reductions in portal pressure and improvement in small-for-size syndrome [444].

Hypoxia

Intrahepatic hypoxia is one of the stimuli which may contribute to the early triggers of liver regeneration[175], *via* a number of mechanisms including the hypoxia induced secretion of complex regeneration promoting molecules from stem cells[176]. Given the critical necessity of maintaining normoxia in other tissues, manipulating pO2 for hepatic regeneration benefits seems an unlikely strategy, however, pharmacological manipulation of HIF has been used to treat renal anaemia[445], and *in vitro* studies suggest that such agents could produce angiogenesis in the liver, as well as a hepatocyte cytoprotective effect[174].

Adrenergic stimulation

The finding that surgical denervation of the liver or pharmacological alpha adrenergic blockade significantly impairs liver regeneration, is consistent with known modulatory effects of catecholamines on secretion of hepatic mitogens (including EGF), as well as the finding that catecholamines increase hepatocyte sensitivity to EGF[251]. Moreover, hepatic stellate cells and hepatic progenitor cells are innervated by the

sympathetic and parasympathetic nervous system[446].

Hepatic sympathectomy is effectively carried out in humans in the context of hilar cholangiocarcinoma resections (when the entire hepatic hilum is skeletonized) and in liver transplantation, when the implanted graft is totally denervated, however there is no obvious clinical evidence that this has a detrimental effect on liver regeneration.

The absence of reported clinical compromise to liver regeneration following sympathectomy in humans may reflect differences in autonomic supply[447], compensatory effects from adrenal catecholamine secretion, or perhaps relatively rapid re-innervation, as seen in some animal models[448]. Finally, the effect of catecholamine stimulation is complicated by the fact that inhibition of alpha adrenergic signals may have a beneficial effect in minimising hepatic stellate cell activation and therefore reduce detrimental excessive fibrosis[446].

The role of platelets and fibrinogen

Platelets clearly have an important role in promoting liver regeneration with animals and humans studies showing impaired liver regeneration in individuals thrombocytopenia[178,179], and the association between thrombocytopenia and post-operative mortality after major liver resection[449]. Moreover, fibrinogen deposition in the liver appears key in driving platelet accumulation in the liver, with post hepatectomy hypofibrinogenaemia being associated with liver dysfunction and mortality. Moreover, studies in rodents suggest platelets are the key source of the auxiliary hepatic mitogen serotonin in liver regeneration, and that serotonin infusion rescues impaired liver regeneration observed in thrombocytopenic mice[256].

Correction of platelet count and fibrinogen levels in patients post hepatectomy has not been investigated in humans, but could potentially have beneficial effects, although thromboembolic risks would have to be taken into account. Serotonin infusion has also not been investigated in humans in the context of liver regeneration, but has been carried out in other clinical contexts to stimulate prolactin[450], and as a desired effect in amitriptyline treatment of depression[451].

Non parenchymal cell modulation

In the setting of liver regeneration with significant hepatic necrosis, the role of non-parenchymal cells may be particularly important. Macrophages clear toxic cellular debris, and mice deficient in macrophage colony stimulating factor 1 (CSF1) which promotes the maturation of macrophages have impaired liver regeneration[372]. Moreover, CSF1 correlates with recovery from paracetamol liver injury in patients with liver failure[373]. In addition to clearing metabolic debris, macrophages may in part act by stimulating the hepatic ductular reaction[452] and limiting fibrosis[453].

Although macrophage colony-stimulating factor-1 has not been used in humans in the context of liver failure and regeneration, it does have a multitude of other clinical applications[454].

Similarly, in rodent models at least, the bone marrow is a significant source of LSEC progenitor cells, which emigrate from the marrow into the bloodstream from which they engraft into regenerating liver, where they produce a significant quantities of the complete hepatic mitogen HGF, in a VEGF driven process[314,315]. Specific LSEC progenitor stimulation therefore offers a theoretical therapeutic opportunity but has not been investigated.

Modifying the proliferative response.

The characterization of the multiple cytokines, hormones and growth factors involved in liver regeneration has motivated investigation of means to modulate the hepatic proliferative response. Such approaches have involved the infusion of specific mitogens, or the use growth enhancing progenitor cells or their secretome and are to date at experimental animal model stage.

These wide-ranging experimental approaches are beyond the scope of this review, but a few examples provide an insight into potential avenues for the future. In a murine model of 85% PH, Cataldegirmen *et al*[455] investigated the potential therapeutic opportunity of blocking the Receptor for advanced glycation end-products (RAGE), which is upregulated in massive hepatectomy and associated with cell stress when binding its ligands. Blockage of RAGE pharmacologically or by transgenic means resulted in significant improvement in survival post massive PH[455]. In a mouse model of partial biliary ligation, Mangieri *et al*[456] report improved liver regeneration produced by infusion of the complete mitogen HGF. Similarly, but targeting the other complete mitogen pathway of the EGFR ligands, Zimmers *et al*[377] demonstrated improved liver regeneration after plasmid delivery of EGF receptor.

In addition to the focus on individual hepatic mitogens, investigation has also been carried out in the infusion of whole cells[457], ranging from primary human hepatocytes[458], pluripotent stem cell derived hepatocyte-like cells[459], and mesenchymal stem cells[460]. Cell based therapies have met several obstacles including sourcing, immuno-compatibility, and potential malignant transformation, thus motivating research into potential for using the secretome of stem cells, thus obviating the difficulties presented by the whole cell therapies[176,461-463].

CONCLUSION

Liver regeneration is highly complex, and current understanding is based largely on animal and *in vitro* models. The likelihood that not all hepatic mitogens have been identified, the multitude of known ones, the complexity and incomplete understanding of their associated biochemical pathways, the equally complex and poorly understood cross talk between cell types, and our even poorer understanding of the factors that cease liver regeneration all suggest that a comprehensive working understanding of the process is improbable in the foreseeable future. Consequently, specific interventions to influence liver regeneration in the clinical setting are commensurately limited, though allow clinicians to at least optimise conditions for liver regeneration to occur. The implications of this in relation to the treatment of liver tumours are most notably applicable in the context of liver resection for malignancy, where assessment and optimisation of remnant liver function not only increases the proportion of patients eligible for treatment, but also improves patient safety. The increasingly sophisticated *in vitro* organoid models, and potential opportunities presented by repopulation of decellularised scaffolds may allow the creation of constructs that allow not only deeper understanding, but also novel therapeutic options.

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REVIEW

Primary vascular tumours of the kidney

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Abstract

Primary vascular tumours of the kidney are rare and may pose diagnostic difficulties because of their similar clinical, morphological, and immunohistochemical features. This article summarizes the clinical and pathological features of primary renal angiosarcoma and anastomosing haemangioma of the kidney including epidemiology, genetics, and prognosis. Renal anastomosing haemangiomas are benign neoplasms characterized by anastomosing capillary-sized vascular channels. These tumours are rare, with about 75 cases reported in the literature. Most anastomosing haemangiomas are found incidentally on ultrasound, computed tomography, or magnetic resonance imaging. Common symptoms include abdominal pain, haematuria, and abdominal mass. Renal anastomosing haemangiomas are characterized by recurrent mutations in *GNAQ* and *GNA14* genes. The prognosis of anastomosing haemangioma is excellent.

Primary renal angiosarcomas are malignant tumours showing endothelial differentiation. To date, 76 cases have been described in the literature. Primary renal angiosarcomas are frequently symptomatic. The clinical features of renal angiosarcomas are similar to those of renal anastomosing haemangiomas, including abdominal pain, haematuria, and abdominal mass. Angiogenesis-related genes and vascular-specific receptor tyrosine kinases such as KDR, TIE1, SNRK, TEK, and FLT1 are upregulated in angiosarcomas. Primary renal angiosarcomas are highly aggressive neoplasms with a poor prognosis despite surgical treatment, chemotherapy, radiotherapy, or targeted therapy.

Key Words: Kidney; Renal tumours; Angiosarcoma; Haemangioma; Anastomosing haemangioma of the kidney; Vascular tumours

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Core Tip: Primary vascular tumours of the kidney are extremely rare. This article

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INTRODUCTION

Although vascular tumours are relatively common in the skin and soft tissue, they are extremely rare in the kidney, ranging from benign to malignant neoplasms that may be diagnostically challenging because of the overlapping clinical, morphological and immunohistochemical features.

These tumours include renal angiosarcomas and renal haemangiomas. Various subtypes of haemangioma have been described in the kidney including cavernous, capillary, and anastomosing haemangiomas[1-4]. However, the most common subtype is anastomosing haemangioma[1,2,5].

This article provides an overview of the clinical and pathological features of anastomosing haemangioma of the kidney and primary angiosarcoma of the kidney, and discusses the epidemiology, genetics, and prognosis.

ANASTOMOSING HAEMANGIOMA OF THE KIDNEY

Renal anastomosing haemangiomas are benign neoplasms characterized by anastomosing capillary-sized vascular channels. These tumours are exceptionally rare with about 75 anastomosing haemangiomas reported in the literature[5-9]. These tumours occur in a wide age range from 10 to 83 years (mean, 49 years) with a male-to-female ratio of 2:1[10].

The aetiology and risk factors for renal anastomosing haemangiomas are unknown. Some cases have been reported in the setting of end stage renal disease[11,12].

The vast majority of anastomosing haemangiomas are found incidentally on radiological evaluation for other purposes. Common symptoms include abdominal pain, haematuria, and abdominal mass[5,10].

The imaging findings are non-specific. On computed tomography, these tumours are often circumscribed, hyperdense, and heterogeneous due to fatty or non-enhancing hypodense areas and show post-contrast enhancement[13].

Renal anastomosing haemangiomas are characterized by recurrent mutations in *GNAQ* and *GNA14* genes[14,15]. *GNAQ* gene encodes guanine nucleotide-binding protein G (q) subunit alpha (Gαq protein) that activates signalling pathways that regulates cell proliferation, survival, development, and function of blood vessels[14-16].

Grossly, anastomosing haemangiomas are typically small ranging from 0.1 cm to 12 cm (mean, 2.2 cm)[5,10,17]. These tumours are often well-demarcated spongy mahogany brown masses[5]. They are usually unilateral and solitary tumours; however, a few cases of bilateral[18] and multifocal[11] tumours have been described.

Histologically, anastomosing haemangiomas consist of anastomosing capillary-sized blood vessels, reminiscent of splenic sinusoids. The blood vessels are lined by bland endothelial cells. Typically, these tumours lack endothelial cell multilayering, papillary tufting, cytologic atypia, necrosis, and prominent mitotic figures. They may show extramedullary haematopoiesis, hyaline globules, and mild lymphocytic infiltrate[1,2,5,10,18].

Rarely, renal anastomosing haemangiomas may infiltrate perinephric fat, renal sinus fat[2,12], and the renal vein or its segmental branches[2,12,18,19]. The neoplastic cells are immunoreactive for CD31, CD34, ERG, FLI1, and factor VIII-related antigen (now rarely used)[5].

Renal anastomosing haemangiomas may co-exist with other renal neoplasms such as metanephric adenoma, papillary adenoma, papillary renal cell carcinoma, acquired cystic disease-associated renal cell carcinoma, and clear cell renal cell carcinoma[11,12].

Most patients with renal anastomosing haemangioma, described in the literature, were treated with radical nephrectomy, reflecting a tendency to overtreat these patients, probably because of inaccurate preoperative diagnosis (Table 1).

The prognosis of anastomosing haemangioma is excellent with no evidence of recurrence, metastasis or tumour-related death at an average follow-up of 24.8 mo (range, < 1-156 mo) (Table 1).

PRIMARY ANGIOSARCOMA OF THE KIDNEY

Renal angiosarcomas are malignant tumours showing endothelial differentiation. These tumours are very rare, with about 76 cases described in the literature as case series and reports[20-24]. There is a male-to-female ratio of 6:1, with patient age ranging from 24 years to 95 years (median, 62 years).

Although angiosarcomas arising in other anatomical sites have been associated with risk factors such as exposure to thorium dioxide, arsenic-based pesticides, polyvinyl chloride, and radiation therapy particularly for breast, endometrial and prostate cancers[25-27], no specific aetiology or risk factors have been established for primary angiosarcoma of the kidney[20,28].

The clinical features of renal angiosarcomas are identical to those of renal anastomosing haemangiomas. Common symptoms include abdominal pain, haematuria, abdominal mass, and weight loss. A rare case of spontaneous tumour rupture with retroperitoneal haematoma has been described[29].

Computed tomography imaging shows large masses with heterogeneous enhancement and hypervascularity[30].

Angiogenesis-related genes and vascular-specific receptor tyrosine kinases such as KDR, TIE1, SNRK, TEK, and FLT1, are upregulated in angiosarcomas[31]. High-level MYC gene amplifications are seen in most radiation-induced and chronic lymphoedema-associated angiosarcomas[32]. A subset of cases is characterized by PLCG1, KDR, and PTPRB mutations[33,34].

Some primary angiosarcomas, typically in young adults, have recurrent CIC gene rearrangements, with or without concurrent CIC mutations, and are characterized by upregulation of CIC-target genes including *ETV1*, *ETV4*, and *ETV5*[34]. Angiosarcomas with CIC gene abnormalities are associated with an inferior disease-free survival[34].

Primary renal angiosarcomas are usually large ranging from 3.6 cm to 30 cm (mean, 13 cm). Typically, they are ill-defined haemorrhagic spongy masses with necrosis[1,20].

Microscopically, these tumours range from well-formed vasoformative areas to areas with solid morphology showing sparse vasoformation. These patterns are often mixed within the same tumour. Vasoformative areas are composed of small to medium-sized anastomosing blood vessels, lined by epithelioid and/or spindled endothelial cells showing nuclear pleomorphism, endothelial papillary tufting, multilayering, intraluminal budding, and hobnailing[1,20,28]. Solid areas consist of sheets of malignant epithelioid and/or spindled cells with subtle vasoformation, cytologic atypia, and mitotic figures. Necrosis may be present. Angiosarcomas usually have a haemorrhagic background and extravasated red blood cells are seen within the tumour[1,20,28].

Epithelioid angiosarcomas are composed of sheets of large atypical polygonal or epithelioid cells with nuclear pleomorphism, high nuclear to cytoplasmic ratio, prominent central nucleoli, mitotic figures, and moderate amounts of cytoplasm. Epithelioid angiosarcomas may be mistaken for carcinoma, melanoma, or lymphoma [20,28,35-37].

The neoplastic cells are positive for CD31, ERG, FLI1, CD34, and factor VIII-related antigen[20,28,38,39]. Epithelioid angiosarcomas may be positive for epithelial markers including CK7, Cam5.2, AE1/AE3, and EMA, which may lead to a misdiagnosis of carcinoma[20].

Similar to angiosarcomas arising at other locations, renal angiosarcomas have a tendency for widespread metastasis at diagnosis or afterwards in the course of the disease. Approximately 66% of patients develop metastases, most commonly to the lung and liver. Other sites of metastasis include bone, lymph nodes, peritoneum, small bowel, soft tissue, and skin. Currently, there are no specific standardized treatment guidelines for primary renal angiosarcomas. These tumours are treated with radical nephrectomy, chemotherapy, radiotherapy, or targeted therapy (Table 2).

Table 1 Treatment, follow-up, and outcome of patients with anastomosing haemangioma of the kidney

Ref.	Treatment	Follow-up (mo)	Outcome
Bean *et al*[14]	Nephrectomy	9	NED
Bean *et al*[14]	Nephrectomy	84	NED
Bean *et al*[14]	Nephrectomy	107	NED
Memmedoğlu and Musayev[41]	Nephrectomy	12	NED
Memmedoğlu and Musayev[41]	Nephrectomy	12	NED
Tahir and Folwell[42]	Nephrectomy	1	NED
Pantelides *et al*[6]	Nephrectomy	6	NED
Downes *et al*[7]	Nephrectomy	NA	NA
Downes *et al*[7]	Biopsy	NA	NA
Chandran *et al*[8]	Nephrectomy	NA	NA
Cha *et al*[9]	Nephrectomy	5	NED
Montgomery and Epstein[2]	Nephrectomy	12	NED
Montgomery and Epstein[2]	Nephrectomy	36	NED
Montgomery and Epstein[2]	Nephrectomy	NA	NA
Montgomery and Epstein[2]	Excision	8	NED
Heidegger *et al*[43]	Nephrectomy	156	NED
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Kryvenko *et al*[12]	Nephrectomy	NA	NA
Al-Maghrabi and Al-Rashed[44]	Partial nephrectomy	12	NED
Caballes *et al*[17]	Nephrectomy	18	NED
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Büttner *et al*[11]	Nephrectomy	NA	NA
Lee *et al*[45]	Nephrectomy	NA	NA
Zhao *et al*[46]	Nephrectomy	12	NED
Kryvenko *et al*[18]	Nephrectomy	7	NED

Kryvenko *et al*[18]	Nephrectomy	6	NED
Kryvenko *et al*[18]	Nephrectomy	3	NED
Kryvenko *et al*[18]	Nephrectomy	122	NED
Tao *et al*[47]	Nephrectomy	21	NED
Abboudi *et al*[48]	Nephrectomy	<1	NED
Silva *et al*[49]	Resection	NA	NA
Berker *et al*[50]	Partial nephrectomy	10	NED
Berker *et al*[50]	Nephrectomy	4	NED
O'Neill *et al*[13]	NA	NA	NA
O'Neill *et al*[13]	NA	NA	NA
O'Neill *et al*[13]	NA	NA	NA
O'Neill *et al*[13]	NA	NA	NA
O'Neill *et al*[13]	NA	NA	NA
O'Neill *et al*[13]	NA	NA	NA
O'Neill *et al*[13]	NA	NA	NA
Brown *et al*[1]	Nephrectomy	72	NED
Brown *et al*[1]	Nephrectomy	24	NED
Brown *et al*[1]	Partial nephrectomy	NA	NA
Brown *et al*[1]	Nephrectomy	24	NED
Brown *et al*[1]	Nephrectomy	NA	NA
Perdiki *et al*[51]	Partial nephrectomy	25	NED
Perdiki *et al*[51]	Nephrectomy	14	NED
Wetherell *et al*[52]	Nephrectomy	1	DFUD
Manohar *et al*[53]	Nephrectomy	24	NED
Omiyale *et al*[19]	Nephrectomy	10	NED
Johnstone *et al*[54]	Nephrectomy	NA	NA
Mehta *et al*[4]	Nephrectomy	3	NED
Mehta *et al*[4]	Nephrectomy	12	NED
Mehta *et al*[4]	Nephrectomy	3	NED
Tran and Pernicone[55]	Nephrectomy	NA	NA
Zhang *et al*[56]	Partial nephrectomy	16	NED
Cheon *et al*[57]	Nephrectomy	6	NED
Chou *et al*[58]	Nephrectomy	8	NED
Chou *et al*[58]	Nephrectomy	14	NED

DFUD: Died from unrelated disease; NED: No evidence of disease; NA: Not available.

Primary renal angiosarcomas are highly aggressive neoplasms with 76.3% of patients dying of tumour within 1 mo to 24 mo (mean, 7.5 mo), despite surgical and adjuvant therapy (Table 2). Poor prognostic factors for angiosarcomas occurring at other anatomic sites include age > 69 years, tumour size ≥ 5 cm, regional disease (*vs* localized disease), non-surgical treatment, and distant metastasis[40].

CONCLUSION

Primary vascular tumours of the kidney are rare neoplasms. Unlike primary renal

Table 2 Treatment, follow-up, and outcome of patients with primary angiosarcoma of the kidney

Ref.	Treatment	Follow-up (mo)	Outcome
Costero-Barrios *et al*[59]	Nephrectomy, Chemo, RT	12	AWD
Peters *et al*[60]	Nephrectomy	2	DOD
Singh *et al*[35]	NA	NA	NA
Kern *et al*[61]	Nephrectomy	3	DOD
Kern *et al*[61]	Nephrectomy	1.5	DOD
Aydogdu *et al*[62]	Nephrectomy	NA	NA
Akkad *et al*[63]	Nephrectomy	30	NED
Witczak *et al*[64]	nephrectomy	NA	NA
Chaabouni *et al*[38]	Nephrectomy	1	DOD
Johnson *et al*[65]	Rapid deterioration	NA	DOD
Zenico *et al*[66]	Nephrectomy	4	DOD
Nguyen *et al*[67]	Nephrectomy, Chemo	18	DOD
Terris *et al*[68]	Nephrectomy, RT	10	DOD
Matter *et al*[69]	Nephrectomy, Chemo, RT	18	DOD
Yoshida *et al*[70]	Nephrectomy, Recombinant IL-2	13	DOD
Pauli and Strutton[71]	Nephrectomy, RT	2	DOD
Martínez-Piñeiro *et al*[72]	Nephrectomy, S	4	DOD
Bernstein *et al*[73]	NA	NA	NA
Liu *et al*[36]	Nephrectomy, RT	6	NED
Yau *et al*[74]	Nephrectomy, Chemo, RT	3	DOD
Carnero López *et al*[75]	Nephrectomy, Chemo	5	DOD
Kazaz *et al*[76]	Nephrectomy, Chemo	NA	NA
Souza *et al*[77]	Nephrectomy	1	DFUD
Detorakis *et al*[78]	Nephrectomy, Chemo	11	DOD
Komoto *et al*[79]	Nephrectomy	9.2	DOD
Boni *et al*[80]	Nephrectomy, Chemo	15	DOD
Chang *et al*[81]	Nephrectomy, Chemo, RT	NA	NA
Iannaci *et al*[82]	Nephrectomy	NA	DOD
Subramanian *et al*[83]	Nephrectomy	NA	NA
Waqas *et al*[84]	Nephrectomy, Chemo	NA	NA
Gourley *et al*[85]	Nephrectomy	NA	DOD
Su[86]	Nephrectomy, Chemo	NA	DOD
López Cubillana *et al*[87]	Nephrectomy, Chemo	5	DOD
Juan *et al*[88]	Nephrectomy, Chemo, RT	9	DOD
Prince[21]	Nephrectomy, RT	NA	A and W
Sesar *et al*[22]	Nephroureterectomy	NA	NA
Testa *et al*[23]	Nephrectomy	27	DFUD
Xuan[24]	Nephrectomy	NA	NA
Brown *et al*[1]	NA	6	DOD
Brown *et al*[1]	NA	11	DOD
Brown *et al*[1]	NA	1	DOD

Brown *et al*[1]	NA	NA	NA
Brown *et al*[1]	NA	1	DOD
Brown *et al*[1]	Nephrectomy	NA	NA
Brown *et al*[1]	Nephrectomy	NA	NA
Brown *et al*[1]	Nephrectomy	2	DFUD
Hiratsuka *et al*[89]	Nephrectomy	29	NED
Adjiman *et al*[90]	Nephrectomy	NA	DOD
Limmer *et al*[91]	Nephrectomy	1	DOD
Darlington *et al*[92]	Nephrectomy, Chemo	12	NED
Allred *et al*[93]	Nephrectomy, Chemo	3	DOD
Fukunaga *et al*[94]	Nephrectomy	13	DOD
Desai *et al*[95]	Nephrectomy, Chemo	4	DOD
Sabharwal *et al*[96]	Nephrectomy, Chemo	>1	NA
Aksoy *et al*[29]	Nephrectomy, S	3	DOD
Heo *et al*[97]	Nephrectomy	NA	NA
Mordkin *et al*[98]	Nephrectomy, Chemo, S	NA	NA
Berretta *et al*[99]	Nephrectomy, Chemo	8	DOD
Lodhi *et al*[100]	Chemo	NA	AWD
Cason *et al*[101]	Nephrectomy, RT	10	DOD
Askari *et al*[102]	Nephrectomy	4	DOD
Guan *et al*[103]	Nephrectomy, Chemo	4	DOD
Papadimitriou *et al*[104]	Nephrectomy	NA	A and W
Celebi *et al*[105]	Nephrectomy, Chemo, TKI, VEGF Inhibitor	13	DOD
Rüb *et al*[106]	Nephrectomy, Chemo	12	AWD
Zhang *et al*[107]	Nephrectomy	NA	NA
Tsuda *et al*[108]	Nephrectomy	21	DOD
Grapsa *et al*[109]	NA	NA	NA
Li *et al*[37]	NA	NA	NA
Qayyum *et al*[110]	Palliative (patient's decision)	NA	NA
Leggio *et al*[30]	Nephrectomy	8	DOD
Garmendia *et al*[111]	Nephrectomy	NA	NA
Sanyal *et al*[112]	Nephrectomy, RT	24	DOD
Cerilli *et al*[113]	Nephrectomy, RT	6	DOD
Douard *et al*[114]	Nephrectomy	3	DOD
Yamamoto *et al*[115]	Nephrectomy, RT	19	NED

RT: Radiotherapy; Chemo: Chemotherapy; TKI: Tyrosine kinase inhibitor; S: Splenectomy; DOD: Died of disease; AWD: Alive with disease; A and W: Alive and well; DFUD: Died from unrelated disease; NED: No evidence of disease; NA: Not available.

angiosarcoma, the prognosis of renal anastomosing haemangioma is excellent with no evidence of recurrence or metastasis. These tumours share similar clinical, morphological and immunohistochemical features, and must be distinguished from each other. Features that favour angiosarcomas include the presence of malignant spindled and/or epithelioid cells with a variable degree of vasoformation, cytologic atypia, prominent mitotic figures, endothelial multilayering, papillary tufting, and necrosis.

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MINIREVIEWS

Detection of circulating tumour cells in colorectal cancer: Emerging techniques and clinical implications

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Abstract

Despite several advances in oncological management of colorectal cancer, morbidity and mortality are still high and devastating. The diagnostic evaluation by endoscopy is cumbersome, which is uncomfortable to many. Because of the intra- and inter-tumour heterogeneity and changing tumour dynamics, which is continuous in nature, the diagnostic biopsy and assessment of the pathological sample are difficult and also not adequate. Late manifestation of the disease and delayed diagnosis may lead to relapse or metastases. One of the keys to improving the outcome is early detection of cancer, ease of technology to detect with uniformity, and its therapeutic implications, which are yet to come. "Liquid biopsy" is currently the most recent area of interest in oncology, which may provide important tools regarding the characterization of the primary tumour and its metastasis as cancer cells shed into the bloodstream even at the early stages of the disease. By using this approach, clinicians may be able to find out information about the tumour at a given time. Any of the following three types of sampling of biological material can be used in the "liquid biopsy". These are circulating tumour cells (CTCs), circulating tumour DNA, and exosomes. The most commonly studied amongst the three is CTCs. CTCs with their different applications and prognostic value has been found useful in colorectal cancer detection and therapeutics. In this review, we will discuss various markers for CTCs, the core tools/techniques for detection, and also important findings of clinical studies in colorectal cancer and its clinical implications.

Key Words: Circulating tumour cells; Colorectal cancer; Tools and techniques; Clinical implications

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Core Tip: Circulating tumour cells (CTCs) in the blood have been found to be mainly associated with the stage of the disease and serve as a prognostic marker for survival in colorectal cancer. Some studies have also reported its role in the diagnosis and treatment monitoring. By focusing molecular research on rare CTCs, targeting cellular markers of CTCs, and discovering new cellular markers may improve the management of colorectal cancer and play a role in prevention of metastatic disease. Patients at high risk might benefit from additional individualized treatment which can be investigated in future clinical trials.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers, which stands second and third in women and men, respectively, across the globe with more than 1.2 × 10^6 new cases and 608700 mortalities annually[1]. It develops due to genetic and epigenetic alterations in human genome and environmental factors. Mode of presentation of CRC can be inherited, familial, and sporadic. Inherited CRC accounts for 5%-10% of all cases, for example, Lynch syndrome, familial adenomatous polyposis, and Peutz-Jeghers syndrome. Among all the CRCs, familial CRC accounts for 20%-30% and sporadic cases approximately 70% of all CRCs which are associated with somatic mutations[2]. There are many invasive and non-invasive diagnostic and prognostic tools with varying sensitivity and specificity, and each has its limitation. There is a need for new tools which may be simpler, non-invasive, cheaper, reproducible, and easily available with high sensitivity and specificity. "Liquid biopsy" is currently the most recent area of interest in oncology, which may provide important tools regarding the characterization of the primary tumour as well as metastasis because tumour cells shed into the bloodstream at the early stages of the disease. In "Liquid biopsy", one of the three types of sampling of biological material can be used, which are-circulating tumour cells (CTCs), circulating tumour DNA, and exosomes. CTCs are one of the main components of liquid biopsy, where subsets of tumour cells can disseminate from the primary tumour and intravasate to the circulatory system. CTCs are non-invasive and safe in comparison to traditional tissue biopsy, and can be used for monitoring of tumour progression and tumour response to therapy in real time. CTCs in peripheral blood serve as a source of valuable tumour markers. The present review will describe the main areas of the ongoing investigation on CTCs with particular emphasis on different tools and techniques used for CTC capturing and analysis, and also currently available data of clinical relevance of CTCs.

CTCs

A tumour cell contains millions of cells maintaining genetic mutations driving them to grow, divide, and invade the local tissues. Some cells separate from the edges of a tumour and are released into the bloodstream or lymphatic system. These cells are CTCs. CTCs can also be defined as cells spreading into vasculature by a primary tumour and they keep circulating in the bloodstream of cancer patients[3]. It was Ashworth (1869) who reported the CTCs for the first time and described the presence of tumour cells with resemblance to the cells from the primary tumour, in the blood of a patient with metastatic breast carcinoma. Later, in 1955, evidence of the presence of CTCs in the blood of a patient with primary and metastatic carcinoma was found by immunohistochemistry. In 1990, Moss and Sanders in their study found evidence for CTCs in seven out of ten disseminated neuroblastoma patients by immunostaining. In CRC, CTCs were first reported in 1993 with the help of conventional cytology and cytokeratin staining. Tumour cells were isolated from 42 patients who underwent

resection with the help of density gradient centrifugation, immune histological evidence for CTCs was reported in 4 out of 42 patients. Above mentioned studies have showed that tumour cells could be detected by traditional immunochemistry techniques; however, their results were based on small sample size and single-center studies.

Some studies have also reported CTC circulation in the body fluids before metastasizing to other parts of the body even in the early stages of the disease[4,5]. Wang *et al*[6] analysed the prognostic role of CTCs, highlighting the importance of CTC count before and after chemotherapy. They found that the presence of CTCs during chemotherapy is an unfavorable but independent factor and may play a role in deciding overall survival (OS) and survival without disease progression [progression free survival (PFS)] in advanced CRC cases. From this study, it was clear that CTCs in peripheral blood can be used as useful tumour markers. Characterization and early detection of CTCs have been reported to play an important role as a prognostic and predictive factor in different types of solid tumours[7,8]. Many epithelial cancers, including breast, prostate and lung cancers, have also been found to be associated with CTCs[9,10].

Early diagnosis, prediction of prognosis, assessment of recurrent risk, individualized treatment, and treatment with curative intent have focused research in the field of CTCs[11]. CTCs have faced difficulties for years because of their very low number (1–10 cells *per* 10 mL of blood) in many studies, and they have a short half-life which ranges from 1 to 2.4 h in blood[12,13], hence posing difficulty in further study. Their detection, quantification, and characterization of molecular features are also difficult. At present, there are several limitations to available CTC isolation techniques. Moreover, only a very small number of CTCs possess metastatic property[14]. Hence, it is very important to characterize them exactly so as to differentiate the non-metastatic CTCs from metastatic ones. There are several techniques which are described here for isolation and detection of CTCs effectively.

CIRCULATING TUMOUR CELL ISOLATION AND DETECTION METHODS

Basic principles

Investigation of CTCs can provide helpful clinical information. However, as described earlier, blood stream harbors very few CTCs and every single CTC is surrounded by 10^6-10^7 mononuclear white blood cells (WBCs). To isolate CTCs and detect their characteristics, it is crucial to isolate them from whole blood cells.

Although there are several methods described for isolation of CTCs (Table 1), there are only two basic approaches. The first one is isolation methods based on the detection of specific surface markers for CTCs, which is also termed as "label-dependent methods" (or cell surface markers), and the second method is based on physical or biological properties of CTCs, termed as "label-independent methods". These approaches are not based on antibodies or other markers for labeling the cells of interest, but they enrich them by use of the difference of physical properties.

Label-dependent methods

In these methods, CTC isolation is based on specific markers. The majority of label-dependent methods use specific epithelial tissue marker-epithelial cell adhesion molecule (EpCAM). EpCAM is the most commonly used method of capturing CTCs because its expression is virtually universal in the cells of epithelial origin and is absent in blood cells. Cell capture with conjugated antibodies followed by purification of captured cells *via* the magnetic field was initially used to enrich CTCs from the blood of patients with prostate or breast cancer. The CellSearch system (Veridex) is a commercial platform which is based on this feature; CTCs are characterized as a population of EpCAM-captured cells that are confirmed to be negative for CD45 and positive for cytokeratins[15]. Other markers are also used, like human epidermal growth factor receptor 2 (HER2), mucin 1 (MUC1), and cytokeratins[16,17].

The CellSearch CTCs system (Veridex) is commonly used, and in today's scenario it is the gold standard and the only FDA-approved method for CTC detection. It was approved in 2004 for extracting CTCs in metastatic breast cancer[18] and later in colorectal[19] and prostate cancers[20]. Equipment cost ranges from 600000-800000 USD.

The CellSearch CTCs Kit is generally used for the enumerization of CTCs of epithelial origin (CD45-, EpCAM+, and cytokeratins 8+, 18+, and/or 19+) from whole blood sample and works on the principle of anti-EpCAM immunomagnetic

Table 1 Techniques for circulating tumour cell isolation, markers, and their limitations

No.	Name	Property	Markers	Limitations	Ref.
1	CellSearch	Isolation by anti-EpCAM antibody coated immunomagnetic beads	EpCAM, CKs, CD45, DAPI	Only suitable for cancer of epithelial origin but not for that undergoing the EMT Cells are not viable after detection	[48]
2	AdnaTest	Separation by way of anti-EpCAM and anti-MUC1 antibody coated immunomagnetic beads	EpCAM, MUC1, mucin-1, HER2	Possible false-positive finding due to expression of a selection marker being present in other cells other than CTCs Cells are not viable after detection	[49]
3	MACS	Immunomagnetic CTC enrichment by antibodies against cell surface markers	CK19, EpCAM, Her-2, MUC-1 CK7, CK8, CK18, CK19	Lengthy processing time and low sensitivity	[50]
4	MagSweeper (Illumina Inc)	Immunomagnetic isolation of CTC by antibodies against EpCAM and cellsurface markers Captured cells are viable with intact RNA	EpCAM, CD45, DAPI	Less sensitive during the early stages of tumour development	[51]
5	CTC Chip	Utilizes bifurcating traps to capture CTCs, release *via* flow reversal	EpCAM, CKs, CD45, DAPI	Identification of CTCs is lower than other methods	[52]
6	GEM chip	Geometrically enhanced mixing chip structure that allows enhanced capture of CTC on antibody coated surfaces	EpCAM, DAPI, CD45, cytokeratin	Low sensitivity	[53]
7	Onco Quick (Greiner BioOne, Frickenhausen, Germany)	Separation of erythrocytes and some leukocytes from CTC. High sensitivity, Quantification	CCNE2, DKFZp762E1312, EMP2	No morphology confirmation; not really capture CTCs	[54]
8	ISET (Rarecells Diagnostics)	Rapid processing; non-antigen dependent; Filter based approach	CKs, EGFR, VE-cadherin, Ki67	Size-dependent, manual processing	[55]
9	EPISPOT	Removes leukocytes *via* CD45 depletion Can detect viable CTCs	CD45, CK19, mucin-1, cathepsin-D	Problem arises when antigen levels are lower or binding efficiency is reduced	[56]
10	Ficoll + RT-PCR	Separation of CTC based on size dependent enrichment. High Sensitivity	CK-19, HER2, h-MAM, CEA, maspin, GABA A, B726P	No morphology confirmation	[57]
11	Cyttel Method	Negative immune-magnetic selection of WBC (CD45 antibody)-High detection rate	CD45	-	[58]
12	MetaCell	Size-based enrichment and separation for viable CTCs	CK-18, -19, -20, CK-7, EPCAM, MUC1, HER2, EGFR	Lengthy processing time	[59]

MACS: Magnetic-activated Cell Sorting; CTC: Circulating tumour cell; RT-PCR: Real-time polymerase chain reaction.

enrichment. For the CTC enumeration step, the CTC kit has reagents to stain and fix the cells. The protocol has been described in detail by Coumans and Terstappen[21]; EpCAM is unique for epithelial cells and is expressed in most carcinomas in a very strong manner, while its expression is limited to embryonic stem cells in non-epithelial cells[22]. However, EpCAM is not a universal cancer marker. EpCAM expression is quite absent in squamous carcinoma or down-regulated if cancer cells undergo epithelial-to-mesenchymal transition (EMT); such cancer cells can escape the capturing process. A gene expression study on breast cancer showed that EpCAM was down-regulated in mesenchymal lines relative to the epithelial cell lines[23] and EMT-induced breast cancer cells[24].

Despite its high specificity and efficiency, some of the disadvantages of the CellSearch system are: (1) It is only suitable for cancer of epithelial origin but not for cancer cells undergoing EMT; (2) CTCs cannot be further analysed in real-time and live-cell conditions, because CTCs cannot be kept alive for a long time; and (3) The use of expensive antibodies leads to high detection cost.

Other label dependent methods

AdnaTest is the second most common method used for CTC detection after the CellSearch. It is a commercially available positive selection method in which immuno-

magnetic beads are coated with a combination of antibodies for the increased capture and enrichment of CTCs. Through gene expression testing of specific tumour markers in the captured cells and comparison of this with their primary and metastatic tumour equivalents, clinicians may analyze the clinical implications of CTCs. Therefore, it has both diagnostic and prognostic value. CTCs captured by magnetic beads coated with antibodies (EpCAM, MUC-1, *etc*.) are then analyzed by multiplex real-time polymerase chain reaction (RT-PCR) gene panels.

The other techniques which are used for CTC enrichment are as follow: (1) Magnetic-activated cell sorting system: This system works on immunomagnetic CTC enrichment by antibodies against cell surface markers. Magnetic-activated cell sorting offers both positive and negative enrichment for the high-efficient and accurate isolation of CTCs (Clinical value: Prognosis and diagnosis); (2) MagSweeper: This system works on immunomagnetic isolation of CTCs by antibodies against EpCAM and other cell surface markers. It can process large amount of blood (approximately 9 mL/h) and can detect 1–3 CTCs *per* 1 mL of whole blood (Clinical value: Prognosis); (3) GEM chip: This is geometrically enhanced mixing chip that permits increased identification of CTCs on antibody-coated surfaces (Clinical value: Treatment monitoring and prognosis); (4) Onco cell enrichment and extraction: This platform uses microfluidic chip with internal surfaces functionalized with an antibodies group against bio-tumour-associated and mesenchymal markers (Clinical value: Treatment monitoring, prognosis, and diagnosis); (5) Graphene oxide chip: In this platform, graphene oxides (GO) nanosheets are used to capture antibodies against cell surface markers of CTCs with a high sensitivity (Clinical value: Prognosis); (6) Ephesia (CTC-chip): Micromagnetic particles are functionalized with EpCAM antibodies which can be self-assembled in a micro-fluidic platform (Clinical value: Prognosis and diagnosis); (7) Quadrupole magnetic separator: This separator works as negative CTC enrichment after it combines with viscous flow stress and magnetic force for the recovery of unlabelled CTCs (Clinical value: Treatment monitoring, prognosis, and diagnosis); and (8) CTC-iChip: This chip works on lateral displacement, inertial focusing, and magnetophoresis for fast isolation of leukocytes by using anti-CD45 and anti-CD66B antibodies in negative enrichment or EpCAM activated beads for CTC enrichment in positive enrichment of CTCs (Clinical value: Prognosis and diagnosis).

Label independent methods

Many newly studied methods forCTC recognition have been reported[25]. Separation of circulating tumor cells by physical properties, *i.e.*, density gradients and gravity, using microfluidic technology[26,27] have been found to be able to capture CTCs efficiently.

The different tools and techniques described for CTC isolation in this category are as follows: (1) ISET: Filter based isolation and enrichment (Clinical value: Treatment regimen and prognosis); (2) MetaCell system: Size-based enrichment and separation (Clinical value: Diagnosis and prognosis); (3) Parylene filter: Filter based isolation and enrichment (Clinical value: Diagnosis and prognosis); (4) ScreenCellCyto: Filter based size-exclusion separation and enrichment (Clinical value: Diagnosis); (5) Cell sieve: Micofilter based isolation and enrichment (Clinical value: Diagnosis and prognosis); (6) Parsorti technology: Micro fluidic separation of CTC based on their size and deformability (Clinical value: Diagnosis and prognosis); (7) RosetteSep CTC enrichment/CD45 depletion: This is an immuno-density negative selection method for CTCs using tetrameric antibody complexes that identify CD45, CD66 ,and glycophorin on WBCs and red blood cells (RBCs) (Clinical value: Prognosis); (8) Onco Quick: Isolation of RBCs and some leukocytes from CTCs by using filtration through porous membrane followed by density-gradient centrifugation for better CTC enrichment (Clinical value: Prognosis); (9) Cyttel method: Based on the negative immuno-magnetic selection of WBCs (antibody CD45) followed by gradient centrifugation and smearing through slides of isolated CTCs (Clinical value: Prognosis and treatment regimen); (10) AccuCyte-CyteFinder: Automated rapid imaging of single rare cells in CTCs, followed by density-based cell separation method (Clinical value: Prognosis); (11) EPISPOT: Negative enrichment using CD45 depletion (Clinical value: Prognosis); (12) Cyto Track: Use of fluorescently labeled cells against EpCAM and scanned with the help of beam (Clinical value: Prognosis); (13) Fiber optic array scanning technology (FAST) (Clinical value: Prognosis); (14) Image Stream: Immunogenetic sorting of blood followed by flow cytometry and enumeration of CTCs by fluorescent microscopy (Clinical value: Diagnosis); (15) DEPArray: Moving dielectrophoretic cages for cell capture coupled with Sanger sequencing (Clinical value: Tumour monitoring and prognosis); (16) Vortex: CTC extraction using microscale vortices and inertial focusing (Clinical value: Diagnosis, prognosis, and treatment planning); (17) ClearCell FX: CTC

separation based on size using Dean Flow Fractionation (Clinical value: Diagnosis); and (18) qRT-PCR: Separation of CTCs based on size-dependent enrichment using CD45, CK19, and CK20 (Clinical value: Prognosis).

COMPARISON OF CELLSEARCH SYSTEM WITH OTHER TECHNIQUES

The high sensitivity and specificity of CTC detection methods have a great effect in improving patient outcomes. Politaki *et al*[28] have compared CTC detection rates and prognostic significance in breast cancer patients by comparing three commonly used methods including CellSearch, qRT-PCR, and double immunofluorescence (IF) microscopy. They analyzed early diagnosed (n = 200) and metastatic (n = 164) breast cancer patients before the start of adjuvant or first-line chemotherapy. They compared CellSearch system, qRT-PCR for *CK19* mRNA detection, and double IF microscopy by using A45-B/B3 and CD45 antibodies and concluded that patients were more likely to be CTC-positive using the CellSearch (37%) than qRT–PCR (37% *vs* 18.0%, P < 0.001) or IF (37% *vs* 16.9%, P < 0.001). In another study[29], CellSearch was compared with Adna Test and RT-PCR in breast cancer, and it was found that multimarker qRT-PCR showed a superior sensitivity for the detection of CTCs in metastatic breast cancer patients compared with the CellSearch system and the AdnaTest. There is limitation of the assessment by PCR as it provides the number of target transcripts based on the actual number of CTCs present in a sample[30] and does not allow the morphological assessment of cells. Two cell-based detection assays, the CellSearch and Onco-Quick (for density gradient centrifugation), on comparison revealed that the CellSearch was a far more accurate and sensitive method to detect and enumerate CTCs[31].

There is one study by Gervasoni *et al*[32], in which they compared the capacity of three methods, multimarker RT-PCR assay, standardized CellSearch method, and dHPLC-based gene mutation analysis, to detect CTCs in the blood of 20 CRC patients (stage I = 5, stage II = 8, stage III = 6, and stage IV = 1). They found CTC positivity in 75% of samples by RT-PCR, 20% by CellSearch method, and only 14.3% of samples were found to be gene mutated with the presence of CTCs by HPLC method. These results show that out of these three methods tested, multimarker RT-PCR assay provides the maximum probability of CTC detection. Future studies, by using the above three distinct methods for follow-up, may provide more information about the prognostic significance of CTCs detected through single method assay *vs* combination of different assays[32].

CIRCULATING TUMOUR CELLS AND THEIR CLINICAL APPLICATIONS IN COLORECTAL CANCER

CTC characterization and number may be useful in several ways where they can be used both as a prognostic marker for survival as well as prediction of response to cancer treatment[33]. A multivariate analysis[34] demonstrated that CTC count is the strongest prognostic biomarker for patient survival. If the CTC number increases or remains static, the treatment can be deemed to be ineffective, whereas, if CTC number decreases, the treatment may be effective. Several studies have shown that the presence of as few as 3 to 5 CTCs in 7.5 mL of blood is associated with poor PFS and OS rates[35]. Studies with the CellSearch system and others have shown that high numbers of CTCs are associated with lower DS and OS rates[36]. In a study of 413 metastatic CRC patients being treated with first, second, or third-line therapy, patients with a baseline CTC number of more than 3/7.5 mL had significantly poor median PFS (4.4 mo *vs* 7.8 mo, P = 0.004) and OS (9.4 mo *vs* 20.6 mo, P < 0.0001) compared with patients with less than 3 CTCs/7.5 mL[37,38]. CTC evaluation, during treatment, may be used as a prognostic predictive marker to determine progression-free survival (PFS) and OS. The CellSearch system has its own limitation; the method of isolation utilizes EpCAM expression on the cell surface of the tumour, which is expressed in 75% of cancer types. A study by Fang *et al*[39] (2016) analyzed the expression of cell surface markers CD133, CD54, and CD44 with the help of flow cytometry to analyze the correlation between cellular subpopulations and colorectal liver metastasis. They observed that the expression of cellular subpopulations (CD133+, CD54+, and CD44+) was higher in the peripheral blood of CRC liver metastasis in comparison with those with no metastasis (P < 0.001). In a study by Lalmahomed *et al*[40] (2015) on peripheral blood of 151 CRC patients who underwent liver metastasectomy, CTCs were detected

by the CellSearch system after a density-gradient-based enrichment step. They found that CTCs were detected in 75 samples (43%), out of which 16% had 3 CTCs/7.5 mL of blood. Patients with or without detectable CTCs have an almost similar 1-year recurrence rate (47% *vs* 48%, respectively). A similar recurrence rate was also reported with low *vs* high CTC count (< 3 or 3 CTCs/7.5 mL of blood: 50% *vs* 47%, respectively). In their report, no difference was found in disease-free survival and OS among patients with or without CTCs. A report by Shimada *et al*[41] (2012) found that detecting CEA/CK/CD133 mRNA in tumour drainage blood (RT-PCR method) could act as a prognostic marker in patients with Duke's stages B and C CRC. The findings of the CTC isolation techniques and their clinical significance have been given in detail in Table 2. Hendricks *et al*[42] (2020) used qRT-PCR for indirect CTC detection, which was already applied in previous studies on CRC patients and found to have prognostic value. An earlier study by Sastre *et al*[43] (2008) reported that the CellSearch system could identify CTCs in CRC patients and that CTC positive cases were correlated with the stage of the disease (P = 0.005) but there was no significant correlation between CEA levels, tumour locations, grade of differentiation, and lactate dehydrogenase (LDH) levels. A meta-analysis by Katsuno *et al*[44] (2008) of a total of nine studies found that CTC-positive patients (in blood samples by RT-PCR), correlated with lymph node (LN)-positive patients (50%) *vs* LN-negative patients (21%).

Guadagni *et al*[45] (2020) have published a couple of studies about the role of CTC based therapeutic decision making in CRC. In the first study[45], they included 62 patients with advanced unresectable rectal cancer and reported that where the patients were selected for the treatment based on CTCs (HPP/target-therapy group, n = 43); the disease control rate was significantly higher (PFS = 8 mo, OS = 20 mo) as compared to those given systemic chemotherapy (n = 19) based on age, co-morbidity, and performance status (PFS = 4 mo, OS = 8 mo). The second study[46] was performed on 106 advanced unresectable CRC patients. The therapy was decided based on CTCs (HAI/targeted, n = 44), age, and co-morbidity performance status (systemic chemotherapy, n = 62). The authors found that the group where treatment was given based on CTCs had longer PFS and median survival (MS) (PFS = 5 mo, MS = 14 mo) as compared to those given therapy based on age and co-morbidity performance status (PFS = 3 mo, MS = 8.5). Finally, they concluded that CTCs can be used to choose therapeutic options in unresectable CRC.

Inherited or acquired resistance in response to specific treatment can be assessed with CTCs which may also work as pharmacodynamic markers. CTCs have enhanced our knowledge and understanding about the primary mechanisms of cancer metastasis. This understanding may be useful in therapeutic manipulation with the help of new targets. CTCs were evaluated in phase I trial based on their count and the expression of insulin-like growth factor-1 receptor (IGF-1R) to find out their therapeutic applications. The CellSearch system was used, either alone or in combination with docetaxel, to count CTCs in patients treated with monoclonal antibodies against IGF-1R. Positive IGF-1R and CTC response was seen in 23 out of 26 patients. These patients responded better in case of combined treatment than in case of the remaining three patients who were negative for IGF-1R. From these findings, it was concluded that CTCs can be used as a potential marker for the selection of chemotherapy[47].

CHALLENGES IN CIRCULATING TUMOUR CELL IDENTIFICATION

CTC interpretation is quite promising but has limitations such as factors like requirement of large volume of blood, small size of the cancer patient population, and the standard value for comparison (*i.e.*, CellSearch, blood sample, other micro-devices, *etc.*). Till now, many reports have enlightened the prospects for cancer patient monitoring, and for few years researchers have focused on CTCs to explore their biological metastatic property and role in cancer treatment monitoring. Among the several important clinical applications for CTC technology is the correlation of CTC count with OS and PFS as a measure of clinical outcome.

The presence of CTCs in the blood sample is also a major challenge. If they are present, their heterogeneity of unknown extent is also present. Because of this nature, it demands an ongoing diversity in the detection and characterization of CTCs using the present available and upcoming methods in the future.

Table 2 Studies showing postoperative isolation of circulating tumour cells in colorectal cancer–markers, techniques, and clinical implications

No.	Technology	Markers	Number of patients	TNM stage	Correlation	Clinical significance	Ref.
1	CellSearch system	EpCAM	164	I-III	With stage	N/A	[60]
		EpCAM	24	IV	With therapy response	May be used in monitoring response to therapy	[61]
		EpCAM	97	II	With stage	Correlates with stage	[62]
		CD133+, CD54+, CD44+	15 (nmCRC); 95 (mCRC)	I-IV	≥ 5 CTCs were 8 times more likely to develop distant metastasis. CTC counts show good correlation with colorectal neoplasm	Independent prognostic marker for nmCRC	[63]
		hTERT, CK19, CK20, CEA	438	I-III	-	Poor relapse free survival	[64]
		hTERT, CK19, CK20, CEA	157	I-III	With stage	Poor relapse free survival and overall survival	[65]
		Survivin, CK20 and CEA	156	I-III	With stages (Duke's) and lymph node metastasis.	Useful as an adjunct in detection of CRC patients	[66]
		CD133, CEA, CK20, CK19,	197	II-III	CEA/CK/CD133 expression and stage (Duke's)	Prognostic significance (Duke's stages B and C)	[44]
		hTERT, CK-19, CK-20, CEA, GAPDH and mRNA	72	I-IV	CEA, mRNA: With stage, vascular invasion, and postoperative metastasis	Prognostic and predictive	[67]
2	Flow-cytometry with immunofluorescence	CTCs	18	I-III	With stage and also detected in an early cancer stage.	Predictive	[68]
3	Pyrosequencing	KRAS (Codon 12/13)	26	IV	No association	Prognostic	[69]
4	MetaCell separation method	CTCs	98	I-IV	CTC-positive in 83% CTC-negative in 17%	Prognosis and predictive	[70]
5	Mag Sweeper	PIK3CA	242	-	Mutational discordance found between CTCs, DTCs, and metastases, and among CTCs; DTCs from this patient propagated *in vitro* contained a PIK3CA mutation	Investigating new drug therapies	[71]
6	CTC-Chip	EpCAM, HER2, and EGFR	-	-	Efficiency of 87.5%	*In situ* protein expression, and culture CTCs from the same set of cells	[72]

CRC: Colorectal cancer; DTC: Disseminated tumour cells; GAPDH: Glyceraldehydes 3-phosphate dehydrogenase; nmCRC: Non-metastatic CRC; mCRC: Metastatic CRC.

CONCLUSION

CTCs have become a hot pursuit and in recent years many new CTC detection technologies have emerged. Discoveries of these technologies from laboratory to clinical practice are non-trivial. Only a few systems are available for routine use in the clinical setting, but not freely available. CTC detection is challenging because of the small number of circulating cells but has been found both in metastatic and non-metastatic cancer (Table 3). It has been well correlated with the stage of the disease, prognosis, and survival but has a limited role in therapeutic decision-making. There is a need for the development of newer, cheaper techniques of CTC detection which can be used as an alternative to invasive diagnosis and treatment monitoring. Future research is required as the current literature has limited information on its use in routine clinical practice but the future is promising.

Table 3 Circulating tumour cells in metastatic *vs* non-metastatic colorectal cancer

No.	Type of CRC	Markers used	Detection method used	Relevance	Clinical implications	Limitations of the study	Ref.
1	nmCRC	CEA, CA19-9, CA72-4	Cyttel	Diagnostic/prognostic/predictive	Combination of CTCs and CEA: Diagnostic and prognostic indicators	Small sample size, weak power of the study	[73]
2	mCRC	CK, CD45	Immunomagnetic separation	Prognostic/predictive	The number of CTCs before and during treatment is an independent predictor of PFS and OS in patients with mCRC	The baseline unfavourable CTC was low (26%) and overall CTC yield was less than in other epithelial cells	[74]
3	mCRC	ALDH1, CD44, CD133, MRP5, Survivin	qRT-PCR	Prognostic	Poor prognosis and chemo therapy non-responsiveness Survivin and MRP5 selection of mCRC patients resistant to 5-FU and L-OHP	Require further molecular analyses of CTCs for selection of targeted agents	[75]
4	mCRC	CEA	Cyttel method, immunofluorescence *in situ* hybridization technologies (imFISH)	Prognostic	PFS, OS	Small sample size Lack of dynamic enumeration of CTCs	[6]
5	mCRC	VEGF, CD133+, CD34+/KDR + EPC, CD-34 ¯VEGFR2	Flow cytometry/IHC	Prognostic	Treatment response; PFS, OS	-	[76]
6	nmCRC	CD133, CD166, CD44, EpCAM, ALDH1	Tissue microarray, IHC	Prognostic	No association with poor clinical response; OS	Treatment information was missing (local recurrence, distant metastasis, and postoperative therapy)	[77]
7	nmCRC	CK19, MUC1, CD44, CD133, ALDH1	Flow-cytometry, CellSearch, Cytomorphology, qPCR	Prognostic	May be useful as a therapeutic target; PFS, OS	-	[78]

CRC: Colorectal cancer; nmCRC: Non-metastatic CRC; mCRC: Metastatic CRC; OS: Overall survival; PFS: Progression-free survival; IHC: Immunohistochemistry.

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MINIREVIEWS

Immunotherapy combinations and chemotherapy sparing schemes in first line non-small cell lung cancer

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Abstract

In recent years, studies have explored different combinations of immunotherapy and chemotherapy. The rationale behind these is the improved survival outcomes of new immunologic therapies used in first-line-treatment of advanced non-small cell lung cancer. Moreover, for the most-studied combinations of anti-programed death-1 (PD-1)/programed death ligand-1 (PD-L1) with the addition of platinum-based chemotherapy, recent research is investigating whether combining different immunologic antitumoral mechanisms of action, such as anti-PD-1/PD-L1 and anti-CTLA-4, or anti-PD-L1 and anti-TIGIT, with or without chemotherapy, can improve efficacy outcomes compared with more classical combinations, or compared with standard chemotherapy alone. Here, we present the data of the

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main randomized studies that have evaluated these combinations, focusing on the basic rationale behind the different combinations, and the efficacy and tolerability data available to date.

Key Words: Immunotherapy combinations; Anti- programmed cell death protein; Anti-programmed cell death ligand 1; anti-TIGIT; anti-CTLA-4; Combo; Non-small cell lung cancer

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Core Tip: This review presents the results of the main articles reporting on immunotherapy combinations, with or without chemotherapy, focusing principally on efficacy and toxicity data. The convenience of adding shorter chemotherapy regimens *vs* the standard 4-cycle regimens to immunotherapy doublets is discussed.

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INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide. An increased knowledge of lung cancer pathology as well as of tumoral immunity has permitted the discovery of different targeted molecular treatments and immune-therapies involving an important change of the lung cancer paradigm[1].

Anti-programed death-1/programed death ligand-1 (PD-1/PD-L1) treatment, recently approved in advanced lung cancer treatment, is an important immune checkpoint of anti-tumor activity in our immune system. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is another component of these control immune checkpoints and different inhibitors have been developed to look their role in tumor recognition[2] (Figures 1 and 2).

Non-small cell lung cancer (NSCLC) has a high level of heterogeneity secondary to a different molecular immune subtype and a complex immune environment. This complexity justifies heterogenous responses to immunotherapy (IT) and their combinations[2].

The administration of mono-IT (m-IT) with anti-PD-L1/PD-1, or a combination of this type of agent with platinum-based chemotherapy (CT), is the standard therapy for the different subtypes of non-small cell lung cancer without treatable mutations. Several studies focusing on combinations of different checkpoint inhibitors, such as anti-PD-1/PD-L1 and anti-CTLA-4, have shown these to be advantageous compared with CT alone[3,4]. The association of these IT combinations with CT has also been analyzed in two phase III studies [Checkmate 9LA study (CM9LA) and CCTG BR.34], with interesting results in favor of the new combinations[5,6]. The CYTISCAPE study, presented recently in ASCO 2020, proposes a combination of anti-PD-L1 and anti- T-cell immunoreceptor with Ig and ITIM domains (TIGIT), a new checkpoint inhibitor present in activated T-cells. This phase II study reported favorable outcomes for this IT doublet (d-IT) in the population with PD-L1 > 1% *vs* monotherapy with anti-PD-L1[7].

Here, we briefly summarize the most significant aspects of these new combinations.

BIOLOGICAL BASIS OF IT COMBINATIONS

The biological rationale for combining IT is based on the complexity of immune surveillance regulation, the effect of other anti-tumor treatments on tumor immunogenicity, and the non- redundancy of immune checkpoint regulation[8].

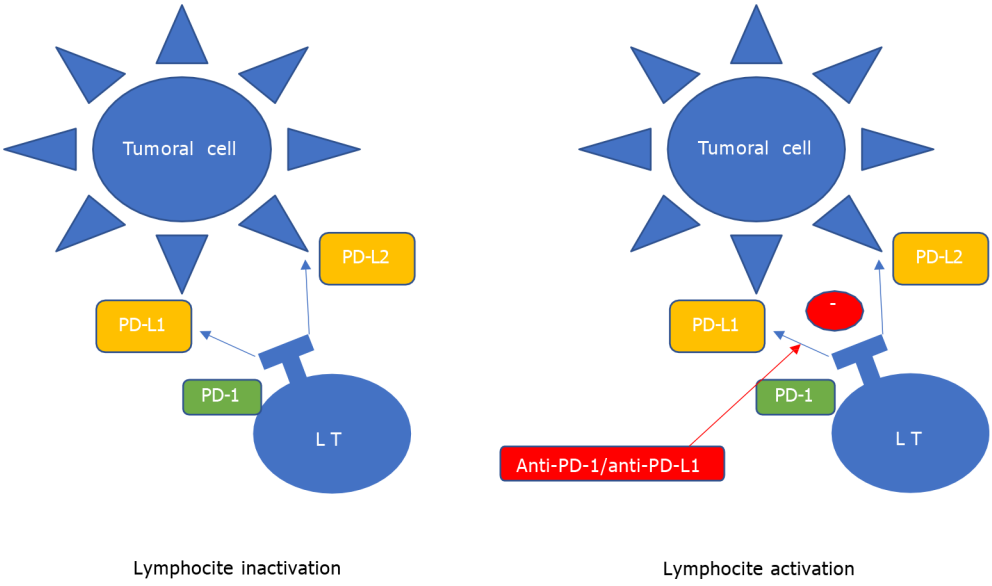


Figure 1 A schematic diagram showing programed death-1/ programed death ligand-1 interaction with immune cells in cancer. PD-1: Programed death-1; PD-LI: Programed death ligand-1.

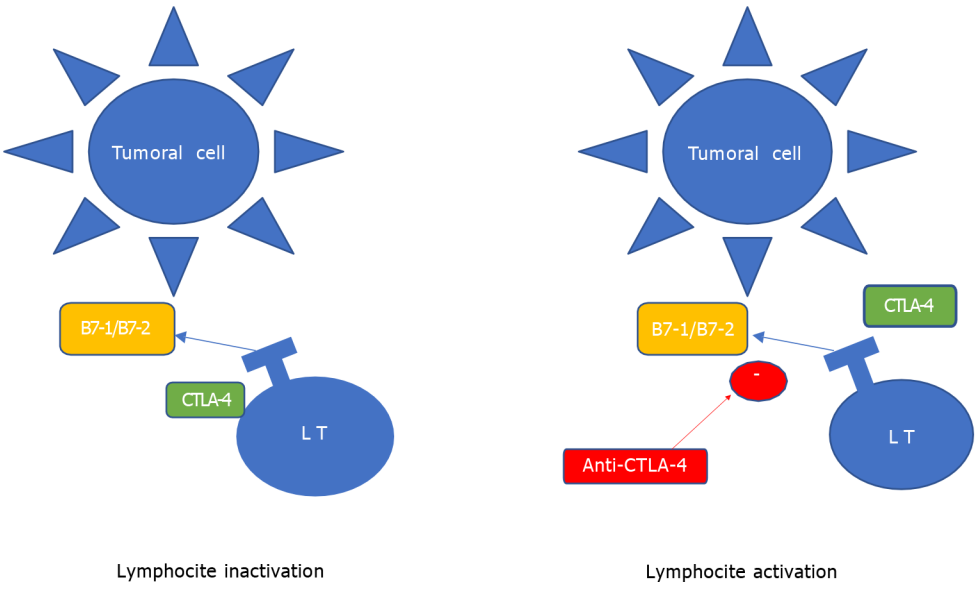


Figure 2 A schematic diagram showing cytotoxic T lymphocyte–associated antigen 4-2/B7-1-B7-2 interaction with immune cells in cancer. CTLA-4: Cytotoxic T lymphocyte–associated antigen 4.

Most chemotherapeutic agents are regarded as immunosuppressants, exerted this effect by a direct inhibition or killing of effector cells, as well as indirectly by inducing anergy or immune paralysis[9]. However, combinations of CT and ICI have a proven synergistic effect: CT aids in fast tumor regression, and a reduction in tumor burden and immune checkpoint blockade may prolong this effect, thus inducing a long-lasting anti-tumor response[10]. In addition, the non-overlapping toxicity profiles of IT and CT render them good candidates for combination strategies.

In addition to immune regulation, angiogenesis is another hallmark of cancer evolution, promoting tumor vascularity to sustain cancer cell metabolism and invasive capacity, and also favors cancer immune escape. Vascular endothelial growth factor, one of the main regulators of angiogenesis, can also induce T-reg cells and inhibit dendritic cell and T-cell maturation, thus inducing an immunosuppressive status, lymphocyte migration to the tumor site, and negatively regulating immune priming [11,12]. Combining anti-angiogenics with immune checkpoint inhibitors could thus be synergic in reducing tumor vascularization and decreasing the immune suppressive microenvironment. It may also have a synergistic effect by boosting tumor-specific

immunity.

Immune checkpoints exert a negative regulatory effect on CD8+ T cells by interacting with their ligands expressed on different cells, such as APC or T cells. Preclinical data indicate that the combination of PD-1 and CTLA-4 receptor blockade may improve antitumor activity. In vitro combinations of nivolumab plus ipilimumab increase IFN-γ production 2- to 7-fold over either agent alone in a mixed lymphocyte reaction (Table 1). The combination was also observed to exert an increased antitumor activity in 3 of 5 syngeneic murine cancer models. In a murine melanoma vaccine model, blockade with either CTLA-4 or PD-1 antibodies increased the proportion of CTLA-4 and PD-1-expressing CD4/CD8 tumor infiltrating T effector cells, and dual blockade increased tumor infiltration of T effector cells and decreased intratumoral T regulatory cells, compared with either agent alone[13].

TIGIT is a novel immune inhibitory receptor. It is a member of the immunoglobulin superfamily and is expressed in a wide variety of human tumors. TIGIT is expressed on the surface of activated T-cell and natural killer (NK)-cell subsets and interacts with high affinity with CD155 [also known as poliovirus receptor (PVR)]. It is also highly correlated with T-cell infiltration and PD-1 expression[14]. Genetic ablation of TIGIT in T cells in mice results in exacerbated T-cell responses in preclinical models of autoimmune diseases and viral infections, demonstrating the role of TIGIT in the inhibition of T-cell responses. Because TIGIT and PD-1 are coordinately expressed by tumor-infiltrating T cells in several human tumors, inhibition of the TIGIT/PVR pathway may complement and potentiate the anti-tumor activity of a PD-L1 pathway inhibitor. In preclinical models, concomitant blockade of both TIGIT and PD-L1/PD-1 pathways demonstrated superior efficacy over the respective single-agent treatments [15].

In conclusion, based on preclinical and clinical studies, there is a strong rationale for combining IT and other agents, and this potentially synergistic effect has been demonstrated with improved outcomes in the clinical setting.

PHASE I-III STUDIES

As explained previously, there is a clear justification for combining different therapeutic strategies. Consequently, several studies have been designed to explore the clinical efficacy of these combinations in the context of advanced NSCLC.

Since pembrolizumab was first approved in first-line therapy of patients with PDL1 > 50%, several studies, which we describe below, have analyzed the combination of anti PD-1/PDL-1 and anti-CTLA-4, with or without the addition of CT[14] (Table 2).

Checkmate 012 (CM012) was the first phase I study to explore the combination of ipilimumab-nivolumab (NI) *vs* nivolumab (N) in CT-naïve patients with advanced NSCLC. This study assessed three alternative regimens with different doses of I and N, with 6 weekly *vs* 12 weekly schemes of I, with tolerability as the primary endpoint. This study confirmed the safety of the combination, predicting a high response rate and improved survival (OS)[17].

With the data from CM012, Checkmate 227 (CM227), a phase III study was proposed. This study was comprised of three parts: Part1A that assessed the efficacy of the combination of NI *vs* N in monotherapy *vs* standard CT in patients with negative PD-L1 expression, part 1B that compared the combination of NI *vs* N + CT *vs* standard CT in PD-L 1 negative patients, and part 2 which evaluated the efficacy of the combination of N + CT, regardless of histology and PD-L1 expression. The study reported a global benefit for the NI doublet in OS, independently of PD-L1 [17.1 ms *vs* 13.9 ms, hazard ratio (HR) 0.73; 95%CI: 0.64-0.84]. When these data are analyzed in relation to PD-L1, several important observations can be made: in the PD-L1 negative group the benefit in OS in favor of the IT doublet was maintained at 17.2 *vs* 12.2 mo (HR 0.62, 95%CI: 0.48-0.78). In the PD-L1 positive group (≥ 1%), OS was also better with the combined IT treatment (17.1 ms *vs* 14.9 ms, HR 0.79; 95%CI: 0.65-0.96). Noteworthily, in this group with 1-49% PD-L1 expression, no differences in OS were found. Also in this study, data of levels of tumor mutational burden (TMB) were reported early on. Hence, in the high TMB group (> 10 mut/Mb), a statistically significant benefit in favor of the combination was reported. Finally, the results of part 2 were communicated, in which the primary final endpoint of OS for N-CT was not reached in patients with non-squamous NSCLC[3].

The CM9LA is a phase III trial with a novel design that adds two cycles of CT to the NI doublet. In the control arm, the CT regimen preferred by the clinician was established, or maintenance with permetrexed in non-squamous histology. With a

Table 1 Main programed death ligand-1 and cytotoxic T lymphocyte–associated antigen 4 pathways

Checkpoint pathways	Anti-PD-L1 pathway	Anti-CTLA-4 pathway
Receptor expression	Activated T-cells	Activated T-cells, B-cells and NK cells
Ligands	B 7.1 (CD80); B7.2 (CD86)	PD-L1 (B7-H1), PD-L2 (B7-DC)
Mechanism of immune modulation	T-cell activation at initial stage; Competition with co-stimulatory receptor CD-28 for ligand binding; Down regulation of helper T cells CD4 activity; Enhancement Tregs-cells immunosuppressive activity	Suppresses activated T cells in tissues and tumor environment; Express in Tregs-cells may enhance immunosuppressive activity; Limits of B-cells and NK- cells activity; PD-L1 interact with CD-80 to down-modulate T-cells activity

NK: Natural killer; PD-L1: Programed death ligand-1; CTLA-4: Cytotoxic T lymphocyte–associated antigen 4.

Table 2 Summary of the main Phase III studies of IT combinations

Trial	Ph[1]	Drugs	Endpoints/outcome	PD-L1/TMB
				Population
				Subgroups
Checkmate 227	III	NI *vs* N *vs* CT[1]	OS PD-L1 > 1% and in non-selected population	PD-L1 ≥ 1%: NI 17 m *vs* CT 14.9 m, *P* = 0.007
			NI *vs* CT	PD-L1 < 1%: NI 17 m *vs* CT 12.2 m, *P* =0.007
			17 m *vs* 13.9 m	
MYSTIC	III	DT *vs* D *vs* CT[1]	OS D *vs* CT OS and PFS DT *vs* CT PD-L1 > 25% population	PD-L1 > 25%: D *vs* CT[1]
				16 m *vs* 12 m, *P* = 0.04
				DT *vs* CT[1] 11.9 *vs* 12 m, *P* = 0.2
				TMB > 20
				DT *vs* CT[1] 21 m *vs* 10 m
Checkmate9LA	III	NI + CT[1] X2 *vs* CT[1] X4	OS in non selected population	NA
			OS NI-CT 15.6 m *vs* CT 10.9 m, *P* = 0.0006	
CCTG BR.34	III	DT+ CT[1] x 4 *vs* Durvalumab + tremelimumab (DT)	OS in non selected population	PD-L1 TPS ≥ 50%, DT-CT *vs* DT (HR 0.64, 95%CI: 0.40-1.04, *P* = 0.07). Plasma TMB < 20 mut/Mb was associated with shorter survival in both treatment groups (HR 1.99, 95%CI: 1.3-3.1)
			16.6 m DT-CT *vs* 14 m DT	
CITYSCAPE	II	TA *vs* PA	ORR and PFS en PD-L1 > 1% PFS TA 7.7 m *vs* PA 3.2 m	PD-L1 > 50%
			ORR TA 37% *vs* PA 20%	PFS TA NA *vs* PA 4.1
				ORR TA 66 % *vs* PA 24%

[1]Ph; Phase.
NI: Nivolumab + Ipilimumab; N: Nivo; DT: Tremelimumab + Durvalumab; D: Durvalumab; PD-1: Programed death-1; TMB: Tumor mutational burden; HR: Hazard ratio; CI: Confidence interval; TA: Tiragolumab + atezolizumab; PA: Placebo + atezolizumab; NA: Not available; CT: Chemotherapy.

follow up of 12.7 mo, the experimental arm showed a significant improvement in OS in all subgroups (15.6 ms *vs* 10.9 ms, HR 0.66; 95%CI: 0.55-0.8)[5].

The combination of Durvalumab (D) and Tremelimumab (T) has also been explored in different phase I-III studies. One of the first phase III trials to analyze this combination in NSCLC was the ARTIC study. This was comprised of two independent sub-studies: one focused on the PD-L1 > 25% population, comparing D *vs* standard CT in patients pre-treated with platinum, and another in the population with PD-L1 < 25% with three arms: DT *vs* D *vs* T in the same patient profile. In both sub-studies, the data from the D and DT arms were more favorable than CT alone[18]. Continuing with this combination of DT in first-line, the MYSTIC phase III study[4] once again focused

on the population of PDL-1 > 25%. The patients were randomized to receive D *vs* DT *vs* platinum-based CT, in this case in first-line of NSCLC. The efficacy data for the mono-IT *vs* CT showed a non-significant superiority for the former (16 mo *vs* 12 mo), although in the d-IT the median OS of 11.9 mo was shorter than that obtained with CT. No benefit in favor of IT was found for PFS either. However, in this study TMB was included as a predictive biomarker of response to IT, with the subgroup TMB > 20 presenting an OS in favor of the IT doublet (21.9 mo *vs* 10 mo).

Continuing to focus on the DT combination, CGC BR.34, another phase III study emerged, in parallel with CM9LA, to address the need to explore the efficacy of d-IT. In this work, 4 CT cycles (platinum-pemetrexed) were added to the DT doublet in first-line therapy in a non-squamous population. Maintenance treatment was allowed in both arms. After a six-month follow up, no differences were found in OS, although the analysis of PFS revealed statistically significant differences in favor of the combined treatment of IT-CT (7.7 mo *vs* 3.2 mo)[6].

CITYSCAPE is a phase II trial that evaluates the possible efficacy of combining Tiragolumab (Ti) (an inhibitor of the immune modulatory receptor TIGIT) with Atezolizumab (A) in first-line of NSCLC with PDL > 1%. Preliminary phase I studies (GO 30103) showed the benefit of combining TIGIT inhibition and anti-PD-L1, revealing a greater response rate in pre-treated patients. The first data from this study were presented in ASCO 2020, and the preliminary analysis was positive in favor of the combined approach (TiA *vs* A-placebo) (5.6 ms *vs* 3.9 ms, HR 0.58; 95%CI: 0.38-0.89). Moreover, an exploratory analysis in patients with high PD-L1 expression, or with a tumor proportion score over 50%, obtained a reduction of 70% in risk of disease progression or death from the illness with TiA *vs* A-P (HR 0.30; 95%CI: 0.15-0.61). This study appears to confirm the effectiveness of combining a dual inhibition of immunotherapeutic mechanisms and their potential benefit in clinical practice, although the final results and the study design of the phase III trial are still pending[7].

WHAT ARE THE BENEFITS OF IT COMBINATIONS TOGETHER WITH SHORT CT CYCLES?

Several preclinical studies have arrived at an interesting rationale to support CT-IT combinations. These combinations are an important challenge in NSCLC treatment, not only in advanced disease, but also in a neoadjuvant setting, where phase III studies such as CM816 have demonstrated a significant benefit of CT-IT pre-surgery compared to classical platinum based CT[19]. Although CT agents are regarded as immunosuppressants by directly inhibiting or killing effector cells, as well as indirectly by inducing anergy or immune paralysis[20], different combinations of CT and ICI have a proven synergistic effect. For example, CT aids in fast tumor regression and a reduction in tumor burden, and immune checkpoint blockade may prolong this effect, thus inducing a long-lasting anti-tumor response[21]. In addition, the non-overlapping toxicity profiles of IT and CT render them good candidates for combination strategies. On the hand, preclinical and clinical investigations have revealed that CT could enhance the efficacy of IT through various mechanisms. CT can induce immunogenic cell death and increase tumor antigen presentation and immune effector infiltration in tumors. Moreover, CT can modulate the immune microenvironment through mechanisms such as increasing cancer cell immunogenicity, enhancing the cytotoxicity of T cells and NK cells, and fostering the accumulation of IFN-γ and tumor necrosis factor (TNF)-α. The latest data show that CT also reduces the number of tumor immune suppressive cells like T regulatory cells, macrophages and neutrophils, and increases the ratio of cytotoxic T-cell/T regulatory cells and PD-L1 expression on tumor cells[22].

Specifically, platinum agents have been shown to partly exert their anti-tumor effect *via* modulation of the immune system. They attenuate STAT6 signaling by blocking STAT6 phosphorylation, resulting in a downregulation of PD-L2 on DCs and tumor cells. This triggers increased tumor cell recognition by T lymphocytes[23]. In a study of NSCLC cell lines, cisplatin upregulated the MHC class I chain-related molecule A and B expression, and led to enhanced NK cell-mediated antitumor effect[24]. Other mechanisms by which platinum agents stimulate the immune system include increasing human leukocyte 1 gene complex expression encoding MHC-I, which is associated with cytotoxic T-cell function[25].

As mentioned in previous sections, a number of clinical trials, such as the one described above, have focused on the administration of combinations of IT in first-line therapy. Overall, it is found that an unblocking of checkpoints of antitumor response

(PD-L1/CTLA-4), using combinations of checkpoint inhibitors, has proved more effective than CT alone or IT in monotherapy. The CM 227 trial, one of the first phase III studies that compared NI *vs* CT, reported a greater OS in the former treatment, regardless of PD-L1 expression[3].

In spite of the overall positivity of the CM 227 trial, an initial crossing of the curves takes place in the first 3-6 mo of follow up, both for the entire study population and also in patients with PDL-1 > 1%. This means that CT would be superior to IT in a profile of patients with more aggressive disease, who would potentially be more chemosensitive.

Another parallel study to CM227 in the PDL-1 > 25% population, also mentioned previously, is the MYSTIC study[4], which compares DT *vs* D *vs* CT. However, the results of this study were more disappointing, reporting no greater superiority for the IT doublet *vs* CT, although DT was more effective in the subgroup with TMB > 20. The CITYSCAPE study positions a new combination of IT (Ti-A) as another option in first-line therapy, and once again PD-L1 is a good predictor correlated with a greater benefit in favor of the combination.

Therefore, in spite of being a new option in first-line, d-IT was no more beneficial than its comparators in any of the studies analyzed, and which have been described here.

Subsequent studies, which aimed at improving the efficacy data of these schemes, incorporated CT regimens of different durations in an attempt to maximize the benefits of these new combinations.

With the goal of compensating for this initial deficit in OS in patients in the CM227 study, the CM9LA study added two cycles of platinum-based CT to the NI combination and achieved a greater benefit in OS for all the subgroups *vs* treatment with CT alone. However, as also reported previously in other studies on IT, no benefit in OS was found for patient smokers or for those aged over 75[5].

Another study that adds CT to the IT doublet is the aforementioned CCTG BR.34 study. This was designed with the same goal, in other words, to try to obtain better outcomes than the MYSTIC study[6]. In this case, efficacy data after a 16-month follow up found no differences in OS (with a non-significant favorable tendency in the PD-L1 > 50% group). However, differences were found in the response rates (28% *vs* 14%) and in PFS 7.7 *vs* 3.2 in favor of the combination with CT. A longer follow-up would probably produce more mature conclusions about the benefits of the IT doublet-CT combination *vs* the comparator with single IT-CT. In this case, in contrast to the MYSTIC study, there are no data to support a benefit in patients with a high TMB, although, once again, the high expression of PD-L1 would seem to predict a greater benefit.

In the debate about combinations of IT and CT, there is some controversy about the number of platinum cycles to use. Hence, the CM9LA study used 2 cycles while in the CCTG BR34 study, 4 cycles were administered. Although there are no data to attribute the difference in results to the number of CT cycles given, it is interesting that in the study with more CT cycles, in which better results would be expected, the data were, in fact, negative. It is, therefore, unclear whether the 2 first cycles are insufficient to compensate for the initial fall in the survival curve due to patients with an early sensitivity to IT.

On the other hand, there is no doubt about the rise in platinum-based toxicity of the longer CT schemes. Therefore, prospective studies may be necessary to determine whether reduced CT schemes, with greater tolerability, in combination with the IT doublet could be at least as effective as standard 4-cycle schemes. Another important aspect to consider about these new combinations concerns the choice of the best biomarker to predict benefits with the IT combination. Compared with PD-L1 expression, in the MYSTIC study higher levels of TMB predicted a greater benefit of the combo-IT. However, these results were not reproduced in other studies that analyzed this marker. For combinations of CT with anti-TIGIT/anti-PD-L1, data have not yet been published.

All these studies have only a short follow up and the results of longer follow up periods are required before we can confirm potential benefits for these new combinations of different and complementary therapeutic strategies.

TOXICITY

The toxicity of combinations with IT, whether this corresponds to d-IT or IT-CT, is highly variable, with cytostatic toxicity in addition to immune-mediated adverse

effects. The rates of grade 3-4 toxicities for the different studies on the d- IT combination are presented in Table 3.

Combinations of IT with the NI scheme in the CM227 trial[3], and with DT in the MYSTIC trial[4], show global rates of side effects of 76.7% and 60.1%, respectively; and of 54.2% in arm D. In both studies, higher rates were obtained for the control arm of CT, of 81.9% in CM227 and 83% in MYSTIC. For toxicity rates of G3 or higher, rates of 32.8% were recorded for the NI combination, 22.9% for the DT combination, 14.9% for D in monotherapy, and 33.8% and 36% in the CT arm, thus confirming in both studies that CT was more toxic than IT. Side effects of G3 or higher resulted in interruption of treatment in 9.4%-12.3% in the combination with the IT doublet, in 3.4%-4.9% in the CT arm and in 4.3% in arm D in monotherapy. As expected, the most frequent toxicities of CT were classical toxicities, such as pancytopenia, especially anemia and G3 neutropenia in 10%, and other common effects such as vomiting and asthenia. Immune-mediated adverse events were reported in 13.6% of arm D in monotherapy and in 28.3% with the DT combination. The most frequent immune-mediated toxicities corresponded to cutaneous reactions (30%-35%) and endocrine events (23%-25%). As published previously in other trials and studies, toxicity was also proportional to the level of PD-L1 expression. In CM227, patients with a level of expression of PD-L1 < 1%, presented less treatment-related G 3-4 toxicity (27.0 in PD-1 < 1% *vs* 32% general population).

As expected, combinations of IT-CT were also associated with an increased toxicity. In the CM 9LA trial[3], the global rate of adverse effects of G3 or higher was 47% in the experimental arm with NI-CT *vs* 38% in the control arm with CT, and slightly higher in the arms with CT of CM227[3] and MYSTIC[4]. In the CCTG BR.34 study[6], the combination of DT-CT presented side effects of G3 or higher in 82%, and in the control arm with DT alone of 70%, with rates of 22.9% reported in the MYSTIC study[4]. It is noteworthy that this toxicity is also higher, if results are extrapolated, than the toxicities of studies using the CT-IT combination such as Keynote 189, where a G3 toxicity of 67.2% was recorded[26].

The most common treatment-related serious adverse events of any grade were more frequent in the experimental group. These included diarrhea, febrile neutropenia, thrombocytopenia and anemia, with rates of 2%-3%. Treatment-related adverse events of severity G 3-4 that led to interruption of treatment were reported in 16%, and were higher than those reported for IT combinations in CM227[3], MYSTIC[4] and Keynote 189[26].

In studies of the doublet IT-CT, the most frequent treatment-related adverse events of any grade, which are commonly associated with CT, were anemia (23%-38%), neutropenia (10%-17%) and thrombocytopenia (5%-10%) in CM 9LA[5]. Grade 3-4 adverse events in the CCTG BR.34 study corresponded to neutropenia (7%-9%), anemia (6%-14%), diarrhea (1%-4%), elevated lipase levels (1%-6%) and febrile neutropenia (3%-4%). In the CM9LA study[3], the commonest immune-mediated adverse events of level G 3-4 were gastrointestinal (6%), cutaneous (4%) and hepatic (4%). Most of these were successfully resolved, with some patients requiring immune modulating medication, including corticosteroids and TNF antagonists. In the CCTG BR 34 trial[4], the incidence of adverse events related to the immune system was similar in all arms (colitis 11%, pneumonitis 6%, endocrinopathy 21%).

The toxicity of the combination of A and Ti was not negligible, (CITYSCAPE)[7], with global rates of side effects of 80.6% *vs* 72%, respectively, and G3 toxicity rates of 19.1% *vs* 14.9%, and rates of interruption of treatment of 10.3% *vs* 7.5% for the control group. Once again, in the experimental arm the most frequent immune-mediated toxicity effects were skin rashes, perfusion reaction, pancreatitis, thyroid alterations and colitis.

The toxicity of the IT combinations is, therefore, greater than that of studies of single IT, at the expense of more immune-mediated effects, as to be expected. Similarly, toxicity increases when CT is added to these IT doublet schemes because of the toxicity associated with platinum regimens. Indirect comparisons of the more classical schemes of m-IT-CT *vs* d-IT-CT show greater toxicity in the latter, directly proportional to the number of CT cycles. Hence, in the decision-support algorithm to select the best first-line scheme in our patients, it is essential to take into account the toxicity profiles of each alternative treatment.

CONCLUSION

The combination of different checkpoint inhibitors, or checkpoint inhibitors combined

Table 3 Summary of the main toxicities in each of the studies on immunotherapy combinations or immunotherapy ± chemotherapy

Phase II-III studies	G3-4 toxicity (%)		Treatment discontinuation (%)	
	Exp arm	Control arm	Exp arm	Control arm
CM 227	32.8 NI	33.8 CT	9.4 NI	3.4 CT
MYSTIC	22.9 DT	36 CT	12.3 DT	4.9 CT
CM9LA	47 NI-CT	38 CT	16 NI-CT	9 CT
CCTG-BG-34	82 DT-CT	70 DT	NA	NA
CITYSCAPE	19 ATir	14 PA	10.3 ATir	7.5 PlA
KEYNOTE-189	67 P-CT	65.8 CT	13.8 P-CT	7.9 CT

NI: Nivolumab + ipilimumab; DT: Durvalumab+ tremelimumab; CT: Chemotherapy; Tir: Tirogolumab; A: Atezolizumab; PL: Placebo; P-CT: Pembrolizumab + chemotherapy; NA: Not available.

with other immune modulating strategies, such as TIGIT inhibition, in the context of first-line therapy in advanced NSCLC produces superior outcomes to classical CT regimens. The addition of CT (of 2 to 4 cycles) to this IT doublet is a new strategy to rescue patients who could initially present a detrimental clinical course after first receiving the IT doublet. It is necessary to continue researching to find more accurate predictive biomarkers of response to optimize the selection of patients who should receive these types of therapeutic strategies.

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MINIREVIEWS

Role of liver transplantation in the management of colorectal liver metastases: Challenges and opportunities

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Abstract

The liver is the most common site of colorectal cancer metastasis. Complete resection of the metastatic tumor is currently the only treatment modality available with a potential for cure. However, only 20% of colorectal liver metastases (CRLM) are considered resectable at the time of presentation. Liver transplantation (LT) has been proposed as an alternative oncologic treatment for patients with unresectable CRLM. This review summarizes the published experiences of LT in the setting of unresectable CRLM from the previous decades and discusses the challenges and future horizons in the field. Contemporary experiences that come mostly from countries with broader access to liver grafts are also explored and their promising findings in terms of overall survival (OS) and disease-free survival (DFS) are outlined along with their study design and methods. The rationale of establishing specific patient selection criteria and the dilemmas around immunosuppressive regimens in patients undergoing LT for CRLM are also highlighted. Additionally, this review describes the findings of studies comparing LT *vs* chemotherapy alone and LT *vs* portal vein embolization plus resection for CRLM in terms of OS and DFS. Last but not least, we present current perspectives and ongoing prospective trials that try to elucidate the role of LT for CRLM.

Key Words: Colorectal cancer; Colorectal liver metastases; Liver transplantation;

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Core Tip: Despite the discouraging results of the previous decades, reports from the recent era showed promising results and reemerged the idea of liver transplantation (LT) for colorectal liver metastases (CRLM). Documentation of patient selection criteria and stronger evidence from ongoing prospective trials may reinforce the implementation of LT as an oncologic treatment for CRLM.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancer entities worldwide, ranking third in terms of incidence, and second in terms of cancer-related death[1]. The overall survival of the patients with CRC depends primarily on cancer staging[2-4]. The liver is the most common site of CRC metastasis, mainly due to its anatomical association with the portal circulation[3]. Published data indicate that approximately 20% of patients with CRC present with concomitant liver metastasis at the first medical consultation, while another 50% develops liver metastasis within the first 3 years after primary tumor diagnosis[2,3,5,6]. The life expectancy of patients with colorectal liver metastasis (CRLM) who do not receive any type of treatment ranges from 12 to 15 mo, and the 5-year survival is less than 5%[2]. Implementation of chemotherapy as the only treatment modality for CRLM results in a median patient survival of approximately 25 mo[7]. Complete tumor resection is currently the only potentially curative treatment with a 5-year and 10-year overall survival (OS) of 38% and 26%, respectively[8]. Yet, only 20% of patients present with a hepatic lesion that can be managed surgically with a curative intent[9-11]. Additionally, what constitutes a surgically resectable CRLM is a matter of debate among surgeons[12,13]. Recent advances in surgical techniques[14] combined with the emergence of newer chemotherapeutic drugs[15,16] have increased the proportion of CRLM amenable to resection. Unfortunately, disease recurrence is still reported in 40%-70% of patients within the first 3 years after surgical excision[17,18] due to the presence of micro-metastatic disease, resulting in a median OS of 10-38 mo for patients relegated to palliative chemotherapy[19,20].

This has led to the consideration of liver transplantation (LT) as an oncologic treatment for patients with CRLM isolated to the liver[9,21]. The aim of this review is to delineate the rationale and outcomes of LT in the setting of unresectable CRLM, and to outline the potential benefits, future perspectives, and ethical dilemmas about this treatment modality.

EARLY EXPERIENCE

LT was historically first attempted in patients with malignant liver tumors (including patients with CRLM)[22]. However, poor survival and high recurrence rates quickly led to restriction of LT utilization to early-stage hepatocellular carcinoma (HCC). The experience gained through the years along with advances in surgical technique and neoadjuvant modalities have broadened the spectrum of malignant indications for LT including advanced HCC, hilar cholangiocarcinoma, as well as metastatic liver tumors (*e.g.*, neuroendocrine metastasis)[23]. This motivated some groups in 1990s to re-assess the role of LT for unresectable CRLM.

The first report of LT for CRLM was from Medical University of Vienna, Austria [24]. Mühlbacher *et al*[24] reported a series of 25 patients who underwent LT for CRLM between 1982-1994 (all patients had lymph node negative disease). In this study, the 1-,

3-, and 5-year post-LT OS was 76% (19/25), 32% (8/25), and 12% (3/25), respectively [19,24]. It should be acknowledged, that after retrospective histological examination of the excisional specimens, lymph node micro-metastases were observed in 15 out of 21 patients who were initially classified as having negative lymph node status. This finding was associated with a decreased post-LT median survival of 28 mo compared to a median survival of 118 mo in patients without micro-metastases[24-26]. Another early experience was published in 1991 by Penn *et al*[27] from University of Cincinnati reporting on 10 patients undergoing LT for CRLM (eight of them due to unresectable tumor and two of them due to chemotherapy adverse effects) with a 70% recurrence rate. Additionally, Pichlmayr *et al*[28] published another series of patients undergoing LT in Germany during 1972-1995, and amongst the reported cases there were 4 patients who underwent LT for CRLM. Two of these patients died in the early post-operative period (one due to acute graft rejection), while the other two patients died from disease recurrence at 11 mo and 33 mo follow-up[28]. The discouraging results from these studies in addition to the lack of standardized criteria for patient selection led to CRLM being established as a formal contraindication for LT over the next decades.

RECENT ERA

The broader access to deceased donor liver grafts in Norway led a group from Oslo University Hospital to investigate the outcomes of well-selected LT candidates with unresectable liver-only CRLM[9,21,29,30]. The first prospective study (SECA-I) was published in 2013 and included 21 patients who had undergone LT from 2006 to 2011 [30]. Inclusion criteria were total resection of the primary tumor, ECOG score 0 or 1, at least 6 wk of neoadjuvant chemotherapy, and absence of extrahepatic disease[30]. Liver resection prior to LT had been performed in 3 patients. The median follow-up time was 27 (range, 8-60) months and the 1-, 3-, and 5-year OS was 95%, 68%, and 60%, respectively. All patients received sirolimus for immunosuppression and none of them received adjuvant chemotherapy. Disease free survival (DFS) was 35% at 1 year[30]. Another publication from the same group reported a total of 19 recurrences in 21 patients (16 were single-site and 3 were multiple-sites at first presentation)[29]. Thirteen of the 16 recurrences were isolated to the lung and patients with isolated pulmonary metastases had a 5-year survival of 72% after recurrence was diagnosed [29]. Notably, there was no isolated hepatic recurrence at initial presentation[29]. However, seven patients developed metastasis to the transplanted liver on subsequent follow-up and six out of those seven patients eventually died from metastatic disease.

Although the results from SECA-I were encouraging, the high recurrence rates led to more stringent candidate selection criteria in SECA-II. SECA-II included 15 patients who had undergone LT for unresectable liver-only CRLM from 2012 to 2016[9]. Similar to the SECA-I trial, all patients received sirolimus for immunosuppression and none of them received adjuvant chemotherapy. The stricter selection criteria required that isolated liver-only CRLM was confirmed by 18F-fluorodeoxyglucose (18F-FDG) positron emission tomography (PET)/computed tomography (CT) scan and patients had more than one year time span from diagnosis of CRC to LT. Additionally, at least 10% response on chemotherapy (according to RECIST-criteria) was a prerequisite for inclusion in the SECA-II study[9]. Resection prior to LT was performed in 4 patients. Median follow up was 36 mo. Compared to SECA-I, the more restrictive selection criteria led to improved 1-, 3-, and 5-year OS of 100%, 83%, and 83%, respectively. However, median DFS remained low at 13.7 mo. Overall, 8 patients were reported to have disease recurrence after LT and 6/8 presented with isolated pulmonary metastasis. On follow-up, 13 patients were alive, and 2 patients died 26 mo after LT due to disease recurrence. The main limitations of this study were the small sample and the relative short follow-up time, but the encouraging results drove the investigators from Oslo to conduct an additional enrollment to the SECA-II study using grafts from extended criteria donors[21]. This study (D-arm of SECA-II) included both patients with synchronous CRLM (within 1 year of primary colorectal tumor diagnosis) and those with concomitant resectable pulmonary metastases or with previously resected pulmonary metastases[21]. Moreover, the investigators did not consider response to chemotherapy as a prerequisite for recruitment[9,21]. Ultimately, 10 patients were included between 2014-2018. The median follow up was 23 mo, the median OS was 18 mo, and the median DFS was 4 mo. Disease recurrence was noted in 8/10 patients with isolated pulmonary metastasis seen in six patients. Overall, five patients were still alive on follow-up with two of them having no relapse at 23 mo and

26 mo after LT[21]. These outcomes established that LT could be considered in patients with unresectable liver-only CRLM only under strict selection criteria.

An international, multicenter, retrospective study of 12 patients was published by Toso *et al*[31] in 2017. Eleven of the patients had received chemotherapy prior to LT. The median follow-up time was 26 mo and the 1-, 3-, and 5-year OS was 83%, 62%, and 50%, respectively, while the 1-, 3-, and 5-year DFS was 56%, 38%, and 38%, respectively. Disease recurrence was noted in six patients with five of them presenting with pulmonary metastasis, while 5 out of the 11 patients were reported to be alive and without evidence of relapse at the end of the follow-up[31]. However, due to the nature of the study patients were not selected according to homogeneous criteria and they were not managed with the same interventions. Despite the limitations, this report demonstrated that LT for CRLM can provide a survival benefit in carefully selected patients, but additional refinement is necessary prior to the broader application of LT as an oncologic treatment for CRLM.

A recent worldwide systematic review and pooled analysis of 110 patients undergoing LT for CRLM reported that the 1-, 3-, and 5-year OS rates were 88.1%, 58.4%, and 50.5%, respectively[32].

Study characteristics and findings for the early experience and recent studies on LT for CRLM are shown in Table 1.

RISK STRATIFICATION CRITERIA

Similar to prior reports establishing specific selection criteria for other liver malignancies (Milan criteria for HCC and Mayo Clinic criteria for hilar cholangiocarcinoma)[33,34] the SECA studies introduced the Oslo score which was used a surrogate marker for favorable prognosis[9,21,30]. One point was assigned for each of the following characteristics: (1) Lesion larger than 5.5 cm; (2) Pre-LT plasma carcinoembryonic antigen (CEA) level above 80 μg/L; (3) Time from primary tumor resection to LT less than 24 mo; and (4) Disease progression while on pre-LT chemotherapy. Each of these factors was significantly associated with poorer OS and the five patients who possessed all four factors comprised five of the six mortalities in the SECA-I trial[30]. Risk stratification was also done utilizing the Fong Clinical Risk Score (FCRS)[35], in which one point was given for the following: (1) Synchronous CRLM (less than 12 mo from diagnosis); (2) Primary tumor with positive lymph nodes; (3) More than one lesion; (4) Tumor larger than 5 cm; and (5) CEA level higher than 200 μg/L. FCRS of 1-2 at the time of diagnosis was associated with significantly increased DFS compared to FCRS of 3-4[9].

The importance of stricter patient selection was also highlighted by the differences in OS and DFS between SECA-I and SECA-II studies, where 5-year OS was 60% and the 1-year DFS was 35% for SECA-I, while the 5-year OS was 83% and the 3-year DFS was 35% for SECA-II[9,29]. Smedman *et al*[21] attributed the poorer outcomes in terms of survival and disease recurrence of the D-arm of SECA-II in the significantly higher Oslo and FCRS scores of the patients compared to the patients of SECA-I and primary SECA-II trials[9,21,29,30]. Therefore, it is apparent that strict patient selection criteria and risk stratification are essential for the broader adoption of LT as a life prolonging oncologic treatment for liver-only CRLM.

IMMUNOSUPPRESSION

Immunosuppression is a controversial topic regarding LT for metastatic diseases in terms of achieving a balance between the risk of graft rejection and the risk of disease recurrence[19,32]. That is because attenuation of the native immune response from immunosuppression is essential to prevent graft rejection, however, it may contribute to unfavorable post-LT outcomes in patients with disseminated malignant disease, as it could facilitate disease recurrence. A study that assessed the impact of sirolimus post-LT for HCC, documented that immunosuppression improved the outcomes in the first few years post-LT and had no effect in DFS or OS beyond 5 years post-LT[36]. Notably, sirolimus was the immunosuppressive regimen used in the SECA trials[9,21,29,30]. Data from a study that compared the growth of pulmonary metastasis in patients enrolled in the SECA trials *vs* patients with CRC and lung metastasis who did not receive immunosuppression, reported that there was significant difference between the two groups in terms of the time needed to double tumors' diameter and volume[37]. Moreover, the same study reported that there was no association between

Table 1 Study characteristics and findings

A. Early experience

Author, Yr	Study period	Number of patients	Clinical outcomes
Mühlbacher *et al*[24], 1991	1982-1994	25	1-yr OS: 76%, 3-yr OS: 32%, 5-yr OS: 12%
Penn *et al*[27], 1991	N/A	10	70% recurrence rate
Pichlmayr *et al*[28], 1997	1972-1995	4	2 post-operative mortalities, 2 late mortalities due to recurrence

B. Recent era

Author, Yr	Study period	Number of patients	Follow-up	1-yr OS, %	3-yr OS, %	5-yr OS, %	DFS
Hagness *et al*[30], 2013	2006-2011	21	27 mo	95	68	60	35% at 1st year
Dueland *et al*[41], 2020	2012-2016	15	36 mo	100	83	83	13.7 mo
Smedman *et al*[21], 2020	2014-2018	10	23 mo	N/A	N/A	N/A	4 mo
Toso *et al*[31], 2017	1995-2015	12	26 mo	83	62	50	56% at 1st year

All values reported for continuous variables are expressed in median. OS: Overall survival; DFS: Disease free survival, N/A: Not available.

sirolimus plasma levels and DFS or growth of pulmonary metastases[37]. However, the current level of evidence is relatively low, and future high-quality studies are required to draw solid conclusions for immunosuppressive therapies after LT for CRLM.

LIVER TRANSPLANTATION VERSUS CHEMOTHERAPY

In 2015, Dueland *et al*[38] published a study aiming to outline the differences in OS of patients with CRC and nonresectable CRLM treated by LT or chemotherapy. The investigators compared the SECA-I study with the NORDIC VII study, which was a multicenter randomized three-arm trial investigating the efficacy of cetuximab added to bolus fluorouracil/folinic acid and oxaliplatin[39]. The 21 patients from SECA-I study were compared with the 47 patients from the NORDIC VII study, in terms of DFS and OS. DFS was 8 to 10 mo in both groups[38]. However, the 5-year OS was 56% in patients undergoing LT compared to 9% in patients receiving chemotherapy[38]. The authors attributed this difference to the pattern of disease recurrence. While small and slow growing lung metastases were the most common recurrence pattern in the LT group, patients in the chemotherapy group presented with progression of the nonresectable CRLM, which has a less favorable prognosis[38].

LIVER TRANSPLANTATION VERSUS PORTAL VEIN EMBOLIZATION PLUS LIVER RESECTION

Emerging surgical advances have been proposed to increase the pool of patients with CRLM that can be subjected to liver resection. Implementation of portal vein embolization (PVE) could render initially nonresectable CRLM amenable to resection [40]. Dueland *et al*[41] compared 50 patients enrolled to SECA studies with a matched group of 53 intention-to-treat patients who have undergone PVE to expand the future liver remnant (FLR) and were planned to undergo liver resection (15 patients did not proceed to liver resection due to inadequate FLR or disease progression). Although the data for the whole LT cohort are not presented to clearly appreciate differences compared to the PVE cohort, the authors mentioned that the two groups had similar selection criteria. Additionally, patients were subclassified in two subgroups; the high tumor load (HTL) group was defined as patients having ≥ 9 metastatic tumors or largest tumor diameter ≥ 5.5 cm, while patients with CRLM below the aforementioned limits were included in the low tumor load (LTL) group[41]. The 5-year OS for patients with HTL was 33.4% in the LT arm (n = 29) compared to 6.7% in the PVE arm (n = 15) of the study without any between-group differences regarding tumor burden score. The 5-year OS for patients with LTL was 72.4% in the LT arm (n = 21) compared to 53.1% in the PVE arm (n = 30), while the tumor burden score was significantly higher

in the LT arm. Accounting that there are no randomized controlled trials comparing LT to PVE plus resection in patients with extensive liver-only CRLM, as well as the fact that these two modalities may not necessarily be applicable to the same pool of patients, this study provides some evidence that LT has promising future perspectives in the field of oncologic treatments for CRLM.

CURRENT AND FUTURE PERSPECTIVES

The International Liver Transplantation Society Transplant Oncology Consensus Conference recommendations, based on the findings of SECA trials, suggested that LT could be implemented in patients with unresectable CRLM with only liver involvement and with a maximum tumor diameter ≤ 5.5 cm, pre-LT CEA ≤ 80 μg/L, response to pre-LT chemotherapy, and time interval from diagnosis to LT ≥ 1 years [42]. However, worldwide liver graft scarcity poses an ethical dilemma which is summarized as follows: How will the distribution of existing grafts to patients with CRLM impact patients with imperative need for a graft? In the United States, the Model for End-Stage Liver Disease score is used to prioritize patients for LT based on severity of liver derangements. However, patients with non-resectable CRLM have no portal hypertension or liver disease and thus are handicapped for access to deceased donors. Such patients could be good candidates for living donor liver transplantation (LDLT). Consequently, considering a long-term OS in the order of 60%, LDLT could offer a very good therapeutic alternative to this group of patients without jeopardizing the cadaveric donor pool.

Several trials attempting to elucidate the role of LT in CRLM are currently ongoing. In addition to the SECA-I (NCT00294827 - active, not recruiting: estimated study completion date May 2023) and SECA-II (NCT01479608 - active and recruiting: estimated study completion date December 2027), the Oslo group is also working on the SECA-III study, which aims to assess the efficacy of LT *vs* other therapies (chemotherapy and surgical resection) with a primary outcome of 2-year OS, and the RAPID (NCT02215889) trial, which aims to evaluate the outcomes of recipient left lateral segmentectomy and implantation of donor segments 2 and 3 followed by removal of the remaining recipient liver segments (second stage hepatectomy) at 4 wk post-LT. The LIVERT(W)OHEAL trial (NCT03488953) will evaluate the outcomes of LDLT in both the donors and the recipients. The largest ongoing trial, estimated to eventually enroll approximately 90 patients, is the TRANSMET (NCT02597348) phase III randomized controlled trial and will evaluate the 3-year OS and disease recurrence or progression in patients with CRLM undergoing LT plus chemotherapy *vs* chemotherapy only. Finally, a trial conducted by the Toronto group (NCT02864485), the COLT trial (NCT03803436), the TRIPLETE trial (NCT03231722), and the Swedish SOULMATE trial (NCT04161092) are also ongoing trials that investigate the utilization of LT as an oncologic treatment for CRLM.

Several other perspectives on the assessment for candidacy should also be incorporated into future studies. FDG is widely used to stage and monitor treatment response in metastatic CRC and the use of PET/CT scan to stage patients, as well as to assess response to therapy has been raised as a parameter of interest. Mutational profiling of CRC has been shown to have an impact on patient outcomes[43], and thus the role of selecting patients for LT based on mutational profiling will need to be addressed. Finally, the use of neoadjuvant radiotherapy to the native liver prior to LT to reduce intraoperative shedding of tumor cells during hepatectomy is also under consideration.

CONCLUSION

The SECA studies from Oslo have demonstrated promising results in prolonging survival with the use of LT as an oncologic treatment for carefully selected patients with unresectable liver-only CRLM. Further evidence is currently awaited from ongoing prospective trials in order to better define the role of LT for unresectable CRLM. The addition of unresectable CRLM as an indication for LT represents a paradigm shift and further confirms versatility of the emerging field of transplant oncology.

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ORIGINAL ARTICLE

Basic Study

Increased tensin 4 expression is related to the histological type of gastric cancer

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Abstract

BACKGROUND

Gastric cancer (GC) is one of the most common malignant tumors worldwide. Tensin 4 (TNS4) is an adhesive protein belonging to the tensin family. This protein is located in focal adhesion sites. The *TNS4* gene is considered an oncogene in numerous cancers. This protein plays an important role in adhesion, migration and proliferation of cells.

AIM

To evaluate expression of TNS4 protein in GC tissues and analysis of the clinical and histopathological parameters as well as the overall survival rate of patients.

METHODS

The expression of TNS4 was assessed in 89 patients using immunohistochemistry.

RESULTS

Positive expression of TNS4 was observed in 49 of 89 patients (55.06%). Higher TNS4 expression was more common in GC tumors with a diameter ≥ 5 cm (P = 0.040). We demonstrated that an increase in TNS4 expression was more frequent in tumors of the histological type without mucinous components than in tumors from mucosal cancers (P = 0.023). Furthermore, TNS4 expression was higher in moderately differentiated tumors than in poorly differentiated and non-differentiated tumors (P = 0.002). Increased TNS4 expression was also noted in the intestinal type of GC according to Lauren's classification (P = 0.020). No statistically significant correlation was found between the expression of TNS4 and the

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Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

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overall survival rate of patients.

CONCLUSION
TNS4 expression was significantly higher in tumors with a diameter ≥ 5 cm of the moderately differentiated intestinal type (according to Lauren's classification) of GC without a mucinous component. Therefore, increased TNS4 expression is related to the histological type of GC with a better prognosis.

Key Words: Gastric cancer; Tensin 4; Adhesion proteins; Immunohistochemistry

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Core Tip: Tensin 4 (TNS4) is an adhesive protein belonging to the tensin family that plays an important role in cell adhesion, migration, and proliferation. These processes are important in cancer development and may limit cancer cell growth and improve patient survival. By applying immunohistochemistry, we investigated TNS4 expression in gastric cancer (GC) tissues and discovered that TNS4 expression was significantly higher in tumors with a diameter greater than 5 cm, in tumors of the moderately differentiated intestinal type (according to Lauren's classification) and in GC without a mucinous component. We concluded that enhanced TNS4 expression was associated with the histological type of GC with a better prognosis.

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INTRODUCTION

In 2018, more than one million people were diagnosed with gastric cancer (GC), accounting for 5.7% of all malignant cancers worldwide; this cancer is ranked 5th in terms of incidence. Moreover, 8.2% of patients with GC died, which reveals the aggressive nature of this disease. GC was diagnosed twice as often in men than in women[1]. The development of this cancer is a multistage process, and there are numerous environmental and genetic factors that contribute to its progression, including infection with *Helicobacter pylori* (*H. pylori*) bacteria and Epstein-Barr virus, consumption of excess salt and alcohol or smoking[2]. An alarming phenomenon is the incidence of this type of cancer in young people under 40 years of age, most often as a result of a genetic predisposition. A previous report showed that 30% of patients under 30 had GC in their family history[3]. The diagnosis of this cancer at early stages of its progression is of key importance. Preoperative chemotherapy followed by surgical removal of the tumor mass in early cancer results in a 90% 5-year survival rate [4]. Therefore, it is crucial to search for molecular and biochemical changes typical of early stages of cancer development to inhibit tumor progression and increase the survival rate of patients with this type of cancer.

Tensin 4 (TNS4), also known as C-terminal tensin-like protein, belongs to the tensin family, which also includes tensins 1–3. The *TNS4* gene is located on the long arm of chromosome 17q12-21. The TNS4 protein has a molecular weight of 77 kDa. In terms of structure, TNS4 is composed of the Src homology 2 domain (SH2) and the phosphotyrosine-binding domain (PTB). The SH2 domain allows the TNS4 protein to participate in the transmission of intracellular signals, while the PTB domain binds to membrane integrator receptors. Unlike tensins 1–3, TNS4 does not have an actin-binding domain[5]. Intracellularly, TNS4 is located in focal adhesion sites, where it enables signaling between the extracellular matrix and the cell interior. From a physiological perspective, the expression of TNS4 has been detected in the prostate and placenta[6]. Pathologically increased TNS4 expression appears in cancerous tumors. *TNS4* was originally classified as a suppressor gene in prostate cancer[6], but as research on tensins progressed, its role as an oncogene was demonstrated in

colorectal, breast, colon, esophagus, and lung cancers and thymoma, where this protein was overexpressed[7-15]. TNS4 plays an important role in biological processes connected with carcinogenesis, such as proliferation, migration, cell adhesion and invasiveness[16].

The aim of our study was to evaluate the IHC expression of TNS4 protein in GC (stages I-IV) as well as to assess the relationship between protein expression and selected clinical and pathological parameters and the overall survival rate of patients.

MATERIALS AND METHODS

Study group

The study was conducted on a group of 89 patients diagnosed with GC who were treated surgically in the 2nd Clinical Department of General and Gastroenterological Surgery of the Medical University of Bialystok in the years 2005–2015. The tissue material was obtained from the archives of the Academic Center for Pathomorphological and Genetic-Molecular Diagnostics in Bialystok. The inclusion criterion was diagnosed adenocarcinoma at any stage of its progression; the exclusion criteria were diagnosed squamous cell carcinoma and other non-epithelial cancers, metastases of other cancers to the stomach and the lack of complete medical records. The normal mucous membrane was used as the control tissue. The research was approved by the Bioethics Committee of the Medical University of Bialystok, permission No. R-I-002/29/2019. The study was conducted in accordance with the World Medical Association Declaration of Helsinki for ethical principles for medical research involving human subjects. The characteristics of the study group are presented in Table 1.

Tissue preparations

Tissues collected during the operation were fixed in 4% buffered formalin and embedded in paraffin. Paraffin blocks were then cut on a microtome into approximately 4-μm-thick sections and stained with hematoxylin and eosin. A routine histopathological examination included the assessment of the histological type of cancer, malignancy grade (G), anatomoclinical stage (pT) and presence of lymph node metastases (pN), infiltration of blood and lymphatic vessels, perineural infiltration, peritumoral inflammation and degree of desmoplasia. Moreover, *H. pylori* infection was assessed by Giemsa staining. The following information was selected from the records with histopathological diagnosis: age and sex of patients, diameter and location of the tumor, presence of distant metastases and the type of cancer according to Lauren's classification.

Immunohistochemistry

Immunohistochemical (IHC) staining was performed on 89 GC tissues. Paraffin blocks were cut on a microtome into approximately 4-μm-thick sections and mounted onto silanized slides. The microscopic sections were incubated overnight at 60 °C and then deparaffinized in xylene solutions and hydrated in a series of alcohol solutions of decreasing concentration (2 × 99.9%, 96%, 70%). The next step was blocking endogenous peroxidase activity by using 3% hydrogen peroxide solution (10 min) as well as nonspecific antibody binding by means of horse serum (anti-mouse/rabbit serum produced in horse, Vector Laboratories, Germany) (20 min). In the following step, the sections were incubated with polyclonal anti-TNS4 antibody at a dilution of 1:75 (Biorbyt, orb186458 produced in rabbit) for 30 min at room temperature. The antibody binding sites were visualized with an ImmPress Universal Antibody Polymer Reagent kit (Vector Laboratories, Germany) and ImmPACT DAB chromogen (Vector Laboratories, Germany). Cell nuclei were stained with hematoxylin. The preparations were then dehydrated in a series of alcohol solutions of increasing concentration and overexposed in xylene solutions.

Validation of TNS4 expression detection

We particularly focused on obtaining reliable results of TNS4 IHC staining. To optimize the TNS4 staining procedure, we used positive and negative controls, and selected primary antibody dilutions (1:50, 1:75, 1:100, 1:200) and incubation times (30 min, 60 min, 120 min) were tested. For the control, antigen retrieval was also performed in buffers with pH = 6.0 and pH = 9.0.

Table 1 The characteristics of study group

Parameter	Number of cases
Age	
< 60	29 (32.58%)
≥ 60	60 (67.42%)
Gender	
Female	29 (32.58%)
Male	60 (67.42%)
Tumor diameter	
< 5 cm	18 (20.22%)
≥ 5 cm	71 (79.78%)
Tumor localization	
Upper 1/3	17 (19.10%)
Middle1/3	33 (37.08%)
Lower 1/3	16 (17.98%)
Whole stomach	23 (25.84%)
Histological type	
Adenocarcinoma	54 (60.67%)
Adenocarcinoma mucinosum	35 (39.33%)
Histological differentiation	
Moderately differentiated	25 (28.09%)
Poorly differentiated	35 (39.33%)
Non differentiated	29 (32.58%)
Depth of invasion	
T1	6 (6.74%)
T2	7 (7.87%)
T3	66 (74.16%)
T4	10 (11.23%)
Lymph node metastasis	
Absent	17 (19.10%)
Present	72 (80.90%)
Distant metastasis	
Absent	61 (68.54%)
Present	28 (31.46%)
Blood vessel infiltration	
Absent	48 (84.21%)
Present	9 (15.79%)
Lymphatic vessel infiltration	
Absent	21 (31.82%)
Present	45 (68.18%)
Perineural infiltration	
Absent	28 (34.15%)
Present	54 (65.85%)

Peritumoral inflammation	
Absent	42 (50.00%)
Present	42 (50.00%)
Desmoplasia	
Small	55 (66.27%)
Large	28 (33.73%)
Helicobacter pylori infection	
Absent	61 (73.49%)
Present	22 (26.51%)
Lauren's classification	
Intestinal	44 (55.00%)
Diffuse	36 (45.00%)

Microscopic evaluation

The preparations were examined under an Olympus BX41 light microscope by two independent pathomorphologists. TNS4 protein expression was evaluated under 400× magnification in 10 representative fields of view. In each field of view, 100 or more cancer cells were assessed. Protein expression was observed in both the cell membrane and the cytoplasm. The presence of TNS4 protein in ≥ 20% of neoplastic cells was considered positive expression.

Statistical analysis

The comparison of TNS4 expression with the selected clinicopathological parameters was carried out by the Mann-Whitney U test for two groups and the Kruskal-Wallis test for 3 or more groups. Dunn's multiple comparison post hoc tests were conducted for the Kruskal-Wallis test. A value of $P < 0.05$ was considered statistically significant. The overall survival analysis was based on the Kaplan-Meier test. The program Statistica 13 (Statsoft, Krakow, Poland) was used for the analysis. Missing data were removed in pairs.

RESULTS

TNS4 expression in GC samples

TNS4 protein expression was tested immunohistochemically in 89 GC samples and 20 normal stomach tissues. Statistical analysis demonstrated that positive TNS4 expression in cancer cells occurred in 49 of 89 patients (55%). In neoplastic cells, the expression of TNS4 was observed in the cell membrane and cytoplasm (Figure 1)

Analysis of the correlation of TNS4 expression with clinical and pathological parameters of GC

The statistical analysis did not show any significant relationships between TNS4 protein expression and the age and sex of patients, tumor location, anatomoclinical stage–pT, presence of lymph node and distant metastases, blood and lymphatic vessel infiltration, perineural infiltration, peritumoral inflammation, desmoplasia or *H. pylori* infection. However, a significant correlation was found between TNS4 expression and tumor diameter. Tumors with a diameter of < 5 cm were present in 20.22% of the patients, while tumors with a diameter of ≥ 5 cm were found in 79.78% of the patients. Positive expression of the TNS4 protein was more frequent in gastric tumors with a diameter of ≥ 5 cm (60.56% of the patients with positive expression) than in tumors < 5 cm in diameter (33.33% of the patients) ($P = 0.040$) (Figure 2A). Furthermore, a significant correlation with the histological type of tumor was demonstrated. Cancers without mucinous components were diagnosed in 60.67% of the patients, and mucosal tumors were diagnosed in 39.33% of the patients. A positive reaction to the TNS4 protein was considerably more frequent in the histological type without a mucinous component (64.81% of patients) than in mucinous adenocarcinomas (40.00% of patients) ($P = 0.023$) (Figure 2B). Statistical analysis also showed a significant

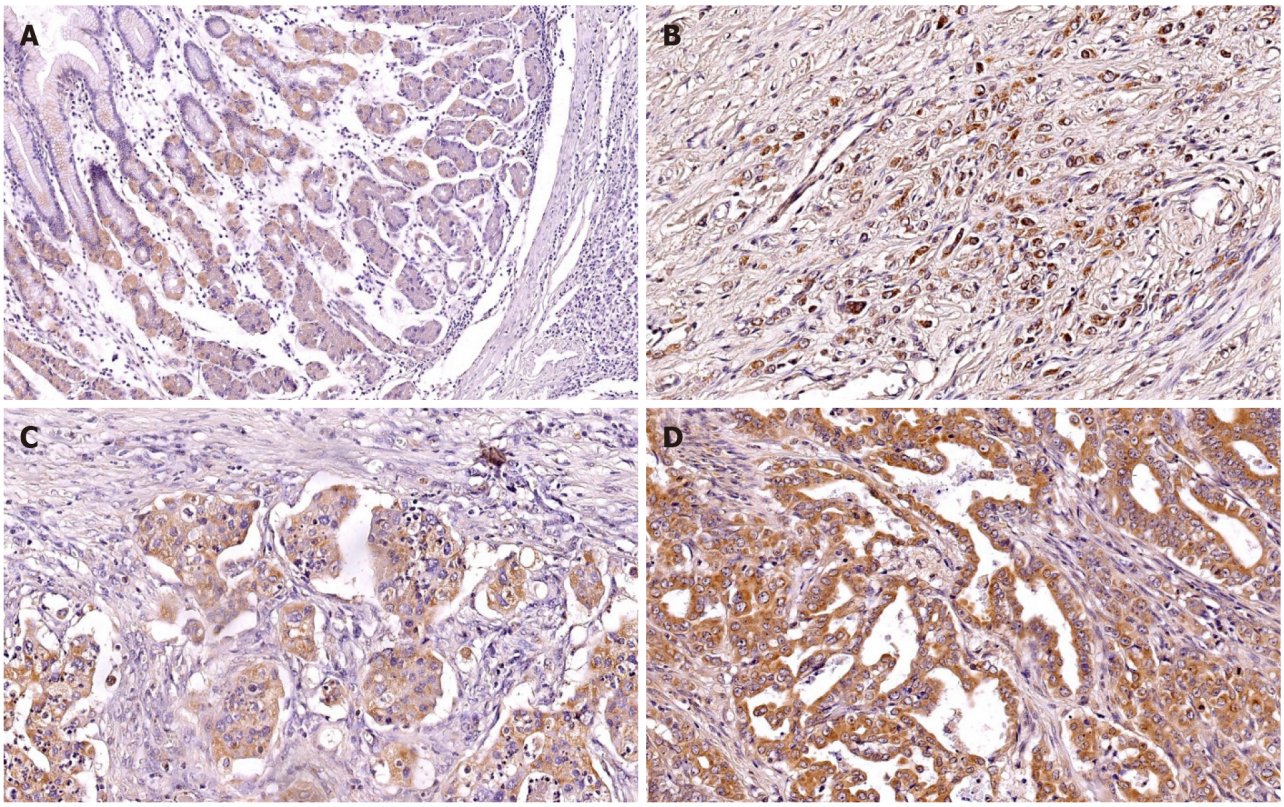


Figure 1 Tensin 4 expression in normal gastric mucosa. A: 100× magnification and gastric cancer samples; B and C: Poorly differentiated gastric cancer (GC); D: Moderately differentiated GC (magnification 200×).

correlation with the malignancy grade. In the study group, there were 28.09% moderately differentiated, 39.32% poorly differentiated and 32.59% non-differentiated tumors. The TNS4 protein occurred much more frequently in moderately differentiated cancers (80.00% of the patients) than in poorly differentiated (57.14%) and non-differentiated (31.03%) tumors (P = 0.002) (Figure 2C). It was also proven that TNS4 protein expression correlates with the type of tumor according to Lauren's classification. The intestinal type was present in 55% of the patients, while 45% of the patients suffered from the diffuse type of GC. The presence of TNS4 was more frequently observed in intestinal-type tumors (70.45%) than in diffuse-type tumors (44.44%) (P = 0.020) (Figure 2D).

Table 2 presents a summary of the correlation between clinicopathological parameters and TNS4 protein expression found in our study.

Analysis of the correlation of TNS4 protein expression with the overall survival rate

Statistical analysis did not reveal a significant correlation between TNS4 protein expression and overall patient survival (P = 0.25) (Figure 3).

DISCUSSION

In our study, we performed an IHC assessment of the expression of the TNS4 protein in GC patients diagnosed with stage I to IV disease. We observed that TNS4 expression was higher in tumor cells than in normal tissue, which is consistent with the results presented by Qi *et al*[17]. Sawazaki *et al*[18] evaluated the clinical significance of *TNS4* mRNA expression in GC and demonstrated that its expression is significantly higher in tumor tissue, as confirmed by Sakashita *et al*[19]. Based on these observations, it can be concluded that changes in the expression of the *TNS4* gene occur in tumor cells compared to normal gastric mucosa cells. This change is reflected in increased expression of TNS4 protein in GC.

In our research, we demonstrated that positive expression of TNS4 protein occurs twice as often in GC tumors with a diameter of ≥ 5 cm than in tumors with a diameter smaller than 5 cm. Similar observations were reported by other authors who noted that TNS4 protein expression increases with the size of breast tumors and hepatocellular

Table 2 Correlation between clinical pathological parameters and tensin 4 protein expression

Parameter	Expression of tensin 4 protein		[a]*P* value
	Negative	Positive	
Age			0.666
< 60	14 (48.28%)	15 (51.72%)	
≥ 60	26 (43.33%)	34 (56.67%)	
Gender			0.182
Female	16 (55.17%)	13 (44.83%)	
Male	24 (40.00%)	36 (60.00%)	
Tumor diameter			0.040
< 5 cm	12 (66.67%)	6 (33.33%)	
≥ 5 cm	28 (39.44%)	43 (60.56%)	
Tumor localization			0.081
Upper 1/3	10 (58.82%)	7 (41.18%)	
Middle1/3	9 (27.27%)	24 (72.73%)	
Lower 1/3	9 (56.25%)	7 (43.75%)	
Whole stomach	12 (52.17%)	11 (47.83%)	
Histological type			0.023
Adenocarcinoma	19 (35.19%)	35 (64.81%)	
Adenocarcinoma mucinosum	21 (60.00%)	14 (40.00%)	
Histological differentiation			0.002 (< 0.001)
Moderately differentiated	5 (20.00%)	20 (80.00%)	
Poorly differentiated	15 (42.86%)	20 (57.14%)	
Non differentiated	20 (68.97%)	9 (31.03%)	
Depth of invasion			0.208
T1	5 (83.33%)	1 (16.67%)	
T2	2 (28.57%)	5 (71.43%)	
T3	28 (42.42%)	38 (57.58%)	
T4	5 (50.00%)	5 (50.00%)	
Lymph node metastasis			0.071
Absent	11 (64.71%)	6 (35.29%)	
Present	29 (40.28%)	43 (59.72%)	
Distant metastasis			0.794
Absent	28 (45.90%)	33 (54.10%)	
Present	12 (42.86%)	16 (57.14%)	
Blood vessel infiltration			0.980
Absent	21 (43.75%)	27 (56.25%)	
Present	4 (44.44%)	5 (55.56%)	
Lymphatic vessel infiltration			0.358
Absent	10 (47.62%)	11 (52.38%)	
Present	16 (35.56%)	29 (64.44%)	
Perineural infiltration			0.628
Absent	13 (46.43%)	15 (53.57%)	

Present	22 (40.74%)	32 (59.26%)	
Peritumoral inflammation			0.453
Absent	19 (46.34%)	22 (53.66%)	
Present	16 (38.10%)	26 (61.90%)	
Desmoplasia			0.710
Small	24 (43.64%)	31 (56.36%)	
Large	11 (39.29%)	17 (60.71%)	
Helicobacter pylori infection			0.824
Absent	26 (42.62%)	35 (57.38%)	
Present	10 (45.45%)	12 (54.55%)	
Lauren's classification			0.020
Intestinal	13 (29.55%)	31 (70.45%)	
Diffuse	20 (55.56%)	16 (44.44%)	

[a]In bracket is *P* value before Dunn's multiple comparison post hoc test.

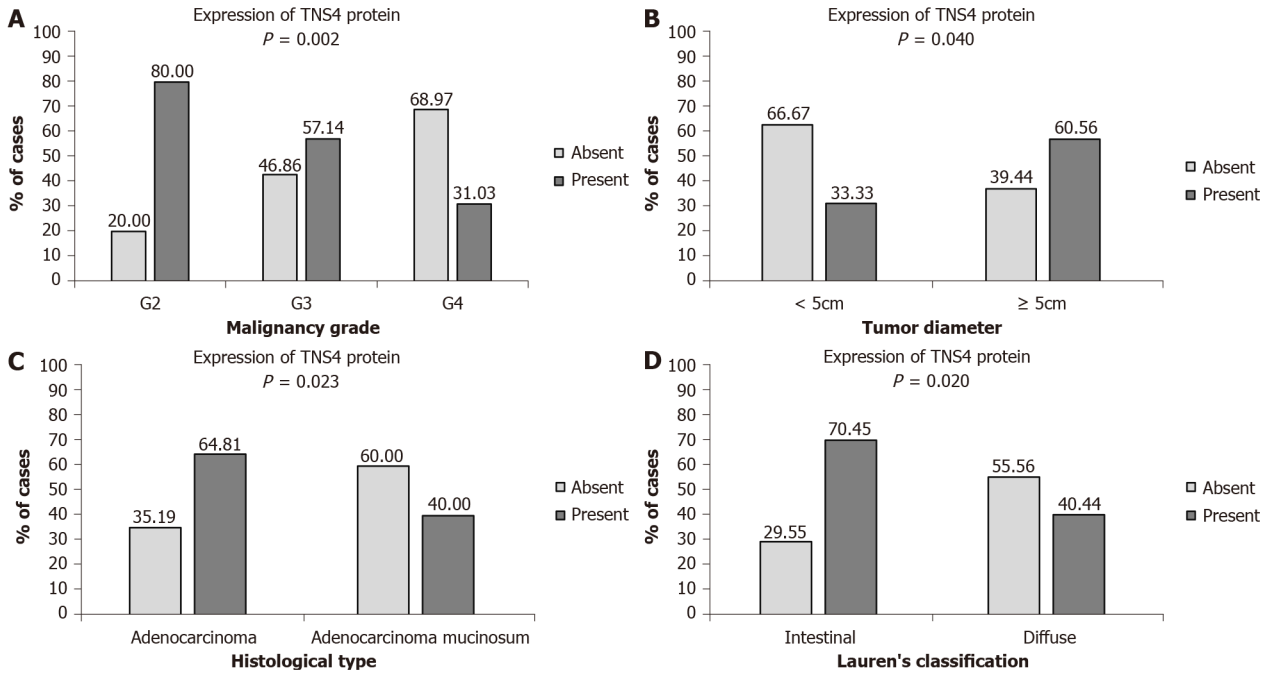


Figure 2 Tensin 4 expression with clinical and pathological parameters of gastric cancer. A: Comparison of tensin 4 (TNS4) expression and (A) malignancy grade, G2 *vs* G4, statistically significant at $P = 0.002$; B: Tumor diameter; C: Histological type; D: Lauren's classification. The comparison of TNS4 expression with the selected clinicopathological parameters was conducted using the Mann-Whitney U test for two groups and the Kruskal-Wallis test for three groups. Dunn's multiple comparison post hoc tests were implemented for the Kruskal-Wallis test. A value of $P < 0.05$ was considered significant. [a]$P < 0.05$ G2 *vs* G4 TNS4 expression level in gastric cancer. G2: Moderately differentiated gastric cancer (GC); G3: Poorly differentiated GC; G4: non-differentiated GC.

carcinoma tumors[11,20]. This finding indicates a correlation between TNS4 expression and the progression of the tumor and may be associated with the activation of signaling pathways, including PI3/AKT and Ras/MAPK, the stimulation of which intensifies the processes of cell proliferation and migration[13]. During the activation of these pathways, TNS4 expression is increased under the influence of epidermal growth factor at the levels of mRNA transcription and protein translation. Increased TNS4 expression leads to enhanced migration and proliferative potential of cancer cells.

We observed significant correlations between the expression of TNS4 and malignancy grade, histological type and type of tumor according to Lauren's classification. TNS4 protein was much more frequently present in moderately differentiated

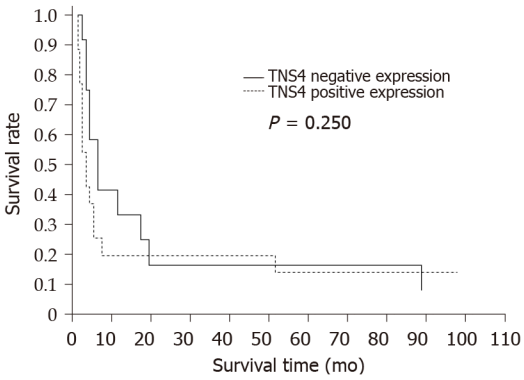


Figure 3 Analysis of the correlation of tensin 4 protein expression with the overall survival rate.

tumors than in poorly differentiated and non-differentiated tumors, in adenocarcinomas without mucinous components and in the intestinal type of GC. The classification of GC based on the degree of glandular cell differentiation is a prognostic factor. The more differentiated the gastric adenocarcinoma, the better the prognosis is for a patient. Poorly and non-differentiated adenocarcinoma cells differ phenotypically from each other, forming clusters without visibly formed glands[21]. There are no reports in the literature suggesting a significant relationship between TNS4 protein expression and the grade of tumor malignancy, which indicates the uniqueness of our study. In contrast, Sakashita *et al*[19] evaluated the *TNS4* mRNA expression and observed that patients with moderately or poorly differentiated GC had higher levels of *TNS4* expression. The differences in the results obtained by these researchers and us may be due to numerous mutations in the *TNS4* gene in tumor cells, entailing changes in the structure of this protein. We demonstrated higher expression of this protein in moderately differentiated gastric adenocarcinoma, which suggests that TNS4 overexpression is associated with a better prognosis of the tumor.

Poorly differentiated GCs are characterized by weak or no developed glandular tubules. Under normal conditions, TNS4 is involved in glandular duct morphogenesis. This phenomenon was described by Wu *et al*[22] after their study on a 3D model of RWPE-1 cells of the prostate. These researchers demonstrated that a decrease in protein expression does not affect the phenotype of the newly formed glands, although their number decreased. The authors suggested that lowering TNS4 expression inhibits the proliferation of normal epithelial cells of the prostate by stopping the cell cycle. However, increased expression of TNS4 leads to disturbances in the formation of glandular ducts through β_1-integrin/FAK pathway induction. Albasri *et al*[8] identified a link between TNS4 and the morphology of colorectal cancer cells. By means of confocal microscopy, these researchers demonstrated that induced expression of TNS4 affects the shape of cancer cells. Under the conditions of TNS4 overexpression, HCT116 cells assume a spindle shape compared to cells with physiologically low expression of TNS4.

In our research, we demonstrated significant differences in TNS4 expression between the two histological types of GC. We noted that positive expression of TNS4 protein was more frequent in GCs without mucinous components than in mucinous adenocarcinomas. Gastric mucosal adenocarcinomas have been linked to poorer prognosis, lower sensitivity to oncological treatment and increased risk of acquiring resistance to chemotherapy[23]. Currently, there are no data available to indicate a link between TNS4 expression and gastric adenocarcinomas without a mucinous component. Phenotypically, GC with a mucinous component is characterized by a considerable amount of mucin in the extracellular space (> 50%)[24].

In our study, we correlated the expression of TNS4 protein with the cancer type according to Lauren's classification. Statistical analysis showed that positive TNS4 staining correlates with the intestinal type of tumor. The presence of this protein was more often observed in adenocarcinomas of the intestinal than diffuse type. The results presented by Chen *et al*[25] indicate that the intestinal type correlates with better prognosis and better clinical and pathological parameters than the diffuse type. From a histological point of view, intestinal-type GC is phenotypically characterized by cells forming glandular ducts in the lumen of which the cancer grows. Usually, these cells do not secrete mucus, but if it is produced, it remains in the lumen of the glands[26]. In the literature, we did not find any studies assessing TNS4 expression in relation to the histological type of GC according to Lauren's classification. The positive

correlation between the expression of TNS4 and the intestinal type of cancer suggests further directions of research as well as the need to investigate the role of this protein in the histogenesis of GC.

Our study did not show any correlation between the level of TNS4 protein expression and tumor progression (pT, pN, pM status), although the literature shows that TNS4 is located in focal adhesion sites and participates in cell migration, thus increasing the metastatic potential of tumor cells. Qi *et al*[17] observed a significant correlation between TNS4 expression and the tumor stage. Their results indicated a correlation between TNS4 expression and deeper infiltrating tumors as well as lymph node and distant metastases. We did not observe similar significant relationships in our study. Other researchers have shown that stimulated TNS4 expression results in an increased ability of cells to migrate, infiltrate into adjacent tissues and metastasize. TNS4 was shown to play an important role in epithelial-mesenchymal transition by stimulating several signaling pathways, including E-cadherin, Akt, Src, TGF-β/Smad and Snail[8,17,27-29]. Other signaling pathways that are stimulated under TNS4 overexpression are PI3K/Akt and Ras/MAPK. Their activation may intensify cell proliferation and migration[13]. Furthermore, *in vitro* studies revealed that TNS4 may promote colony formation, as demonstrated in a study on pancreatic cancer cells[30]. Another important signaling protein in cancer development is MET, which is stabilized by TNS4 and acts as a mediator in carcinogenesis[31]. The exact mechanisms regulating TNS4, *e.g.*, a mutation that stabilizes this protein or amplifies the *TNS4* gene, have not been discovered thus far. Thorpe *et al*[32] reported that TNS4 can be regulated by the EGFR/KRAS and STAT3 pathways. Other studies have indicated that the expression of this protein may be modulated by growth factors and cytokines, *e.g.*, FGF2, PDGF, IGF-1, TGF-β, IL-6 and IL-13[33]. Other literature reports suggest that TNS4 may be engaged in tubulogenesis[34] as one of the stages of angiogenesis. The formation of blood vessel networks by tumor cells is the way in which the tumor microenvironment acquires nutrients and oxygen needed for tumor growth. Various publications have presented evidence for the oncogenic role of TNS4. In our research, we did not confirm the procancer role of the TNS4 protein. Our results prove that TNS4 may be related to the formation of a certain type of cancer, as observed in prostate cancer[35].

Moreover, we did not demonstrate any significant correlations between TNS4 expression level and the patients' overall survival rate. According to the works of Qi *et al*[17] and Sakashita *et al*[19], TNS4 overexpression correlates with shorter survival time and worse prognosis for patients with GC. In our study, the lack of this correlation may result from a small number of evaluated patients and the lack of data on the survival of many of these patients, which is a limitation of our research and does not allow us to draw broader conclusions about the participation of TNS4 in GC carcinogenesis. Additionally, it is possible that differences between the mRNA level in the study by Sakashita *et al*[19] and the protein expression level in our research are because the regulation of TNS4 transcription depends on the type of cells or tissues as well as on potential mutations in the TNS4 gene in GC cells. Thus, the expression level of this protein in different tissues is different. The control of this gene expression may also result from epigenetic modifications[36].

CONCLUSION

The expression of TNS4 was significantly higher in tumors with a diameter of ≥ 5 cm, of a moderately differentiated type of GC without a mucinous component, and of the intestinal type according to Lauren's classification. Increased levels of TNS4 expression are linked to the histological type of GC with a better prognosis.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is still one of the most common malignant neoplasms worldwide in terms of incidence and cancer motility. The development of GC is a multistage process. Tensin 4 (TNS4) belongs to the tensin family. This protein can participate in the transmission of intracellular signals. TNS4 plays an important role in biological processes connected with carcinogenesis, such as proliferation, migration, cell adhesion and invasiveness. It is very important to search for new biomarkers that

could help to diagnose GC at the early stages of its development.

Research motivation

We wanted to evaluate the role of the TNS4 protein in the development of GC. This protein may be a promising biomarker in the diagnosis of GC at the early stages of its development.

Research objectives

The study objective was to show the expression of TNS4 in GC tissues and assess the relationship between protein expression and clinical and pathological parameters and the overall survival rate of patients.

Research methods

In our study, we used immunohistochemistry to evaluate the expression of the TNS4 protein. The research was conducted on a group of 89 patients.

Research results

We observed that higher TNS4 expression was more often observed in GCs with a larger diameter (P = 0.040). Our results also showed that an increase in TNS4 expression was more frequently observed in tumors without mucinous components than in tumors from mucosal cancers (P = 0.023). Furthermore, higher TNS4 expression was demonstrated in moderately differentiated tumors (P = 0.002). Increased TNS4 expression was also noted in the intestinal type of GC according to Lauren's classification (P = 0.020). No significant correlation was found between the expression of TNS4 and the overall survival rate of patients.

Research conclusions

The expression of TNS4 was significantly higher in tumors with a diameter of ≥ 5 cm, of a moderately differentiated type of GC without a mucinous component, and of the intestinal type according to Lauren's classification. Increased levels of TNS4 expression are linked to the histological type of GC with a better prognosis.

Research perspectives

It is possible to develop this study with cell line methods. It will be possible to investigate the potential role of TNS4 in GC cell proliferation.

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ORIGINAL ARTICLE

Basic Study

Tumor irradiation may facilitate the detection of tumor-specific mutations in plasma

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Abstract

BACKGROUND

The mutation-based analysis of circulating tumor DNA (ctDNA) is a promising diagnostic tool for clinical oncology. However, it has low success rate because many cancer patients do not have detectable ctDNA in the bloodstream.

AIM

To evaluate whether preoperative tumor irradiation results in a transient increase of plasma ctDNA concentration due to the induction of apoptosis in radiation-

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exposed cells.

METHODS
This study focused on patients with locally advanced rectal cancer, because preoperative tumor irradiation is a part of their standard treatment plan. Nine subjects, whose tumors contained *KRAS, NRAS* or *BRAF* mutations, donated serial blood samples 1 h prior to the first fraction of irradiation (at baseline), immediately after the first fraction (time 0), and 1, 3, 6, 12, 24, 36, 48, 72 and 96 h after the first fraction. The amount of mutated gene copies was measured by droplet digital PCR.

RESULTS
Five out of nine patients were mutation-negative by ctDNA test at baseline; two of these subjects demonstrated an emergence of the mutated DNA copies in the bloodstream within the follow-up period. There were 4 patients, who had detectable ctDNA in the plasma at the start of the experiment; three of them showed an evident treatment-induced increase of the content of mutated *RAS/RAF* alleles.

CONCLUSION
Local tumor irradiation may facilitate the detection of tumor-specific DNA in the bloodstream. These data justify further assessment of the clinical feasibility of irradiation-assisted liquid biopsy.

Key Words: Liquid biopsy; Rectal cancer; *KRAS*; *BRAF*; Mutations; Tumor response; Radiotherapy

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Core Tip: The detection of circulating tumor DNA (ctDNA) in cancer patients is compromised by the low sensitivity of this assay. We hypothesized that tumor irradiation may lead to the transient increase of ctDNA content due to induction of cell death. Nine patients with locally advanced *RAS*/*RAF*-mutated rectal cancer provided serial blood samples at baseline and during the first 96 h after the first dose of tumor irradiation. Treatment-induced elevation of the concentration of mutated *RAS*/*RAF* alleles in the blood was revealed in five of these subjects. In conclusion, local tumor irradiation may facilitate the detection of plasma ctDNA and thus improve the efficacy of liquid biopsy.

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INTRODUCTION

"Liquid biopsy" is a popular diagnostic tool, which is based on the identification of tumor-specific markers in plasma or other body fluids. The analysis of several proteins, *e.g.*, prostate-specific antigen, carcinoembryonic antigen, CA-125 *etc.* has been utilized for years, however, these assays have significant limitations with regard to specificity and sensitivity[1,2]. The examination of tumor-derived mutations in circulating DNA (ctDNA) is considered to be more promising[3]. Indeed, some methods of genetic testing, for instance, droplet digital PCR (ddPCR) or next-generation sequencing allow the detection of a single mutated allele within a huge excess of wild-type nucleic acids[4,5]. In addition, while the protein-based liquid biopsy is not truly cancer-specific but rather tissue-specific, oncogenic mutations are strongly associated with the malignant phenotype. For the time being, clinical use of

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ctDNA tests is largely limited to the analysis of secondary mutations emerging during targeted therapy, as these assays may help to identify mechanisms of acquired drug resistance and therefore guide the subsequent treatment choice. It is anticipated that in the near future liquid biopsy will support other components of cancer care, such as screening, early diagnosis, analysis of treatment outcome and monitoring of relapse of tumor disease[6-11].

ctDNA-based liquid biopsy may have unacceptably low sensitivity. It appears that many categories of neoplasms (medulloblastomas; gliomas; cancers of kidney, thyroid, breast, *etc.*) are composed of relatively well-preserved cells, which do not shed DNA in the bloodstream, at least when the tumor is small[12,13]. Consequently, the analysis of ctDNA cannot substitute the detection of mutations in tumors tissue, *i.e.*, tissue biopsy cannot be easily replaced by liquid biopsy. The development of tools, which allow non-invasive examination of tumor characteristics, is of great value. One of the options may involve utilization of various agents, which induce tumor cell death and thus facilitate DNA shedding. In particular, tumor irradiation may increase tumor-specific ctDNA level due to the involvement of the above mechanism[14-16].

While considering the appropriate model for the validation of this assumption, we found it reasonable to focus on rectal cancer. Approximately 40%-50% of rectal carcinomas contain missense mutations in *KRAS, NRAS* or *BRAF* oncogenes, which can be used for ctDNA assays[17-19]. Furthermore, many rectal cancer patients undergo preoperative radiotherapy (RT) as a part of the treatment plan. Here we present the results of the study, which involved consecutive patients with mutation-positive rectal cancer. We demonstrate, that tumor irradiation indeed results in a transient increase of concentration of tumor-derived DNA and thus can be considered as a liquid biopsy supporting tool.

MATERIALS AND METHODS

Study workflow

The study considered treatment-naive patients with histologically verified locally advanced rectal cancer (T1-2/N1-2/M0, T3-4/N0-2/M0), who were referred to the St.-Petersburg City Cancer Center between February 2019 and April 2020 and who planned to undergo preoperative RT. The study was approved by the local Ethics Committee. Thirty patients provided informed consent and underwent *RAS/RAF* mutation testing (Figure 1). Thirteen analyzed tumors carried nucleotide substitutions in the mentioned genes. Four subjects failed to participate in the study due to various reasons (two tumors contained "rare" *RAS* mutations (*KRAS A59G* and *NRAS G12C*), which could not be detected by available ddPCR assays; 1 patient experienced rapid disease progression and was not subjected to RT; 1 patient developed acute parotitis and went to another hospital for treatment). Finally, nine patients were included in the ctDNA study and provided serial blood samples. Clinical characteristics of invited patients are summarized in the Supplementary Table 1.

RT and response evaluation

RT was performed according to routine procedures either with 45–50 Gy in 25–28 fractions or short-course radiation therapy (25 Gy in 5 fractions) with or without concurrent fluoropyrimidine-based chemotherapy. Chemotherapy was delivered according to standard regimens (capecitabine 825 mg/m^2 twice daily given within 5 d per week for 6-8 weeks). After RT patients were restaged with magnetic resonance imaging and the response was evaluated according to the TRG (tumor regression grade) system[20]. When the patients were surgically treated, the pathologic response was evaluated according to Mandard criteria[21]. The treatment results summary is presented in Supplementary Table 2.

Sample collection and processing

Patients provided blood at 11 different time points: 1 h before the first fraction of radiation (at baseline), immediately after the first fraction (time 0), and 1, 3, 6, 12, 24, 36, 48, 72 and 96 h after the first fraction (Figure 2). Ten milliliters of blood were collected into PAXgene Blood ccfDNA Tubes (Qiagen) or cf-DNA/cf-RNA Preservative Tubes (Norgen). Plasma samples were separated from the cellular fraction within 2-8 h after the blood-draw by two-step centrifugation (400 g for 10 min at room temperature followed by 14400 *g* for 10 min at 4 °C). The supernatants were aliquoted into 2 mL tubes and stored at −70 °C until further use. Cell-free DNA was extracted with the QIAmp Circulating Nucleic Acid Kit (Qiagen) as recommended by

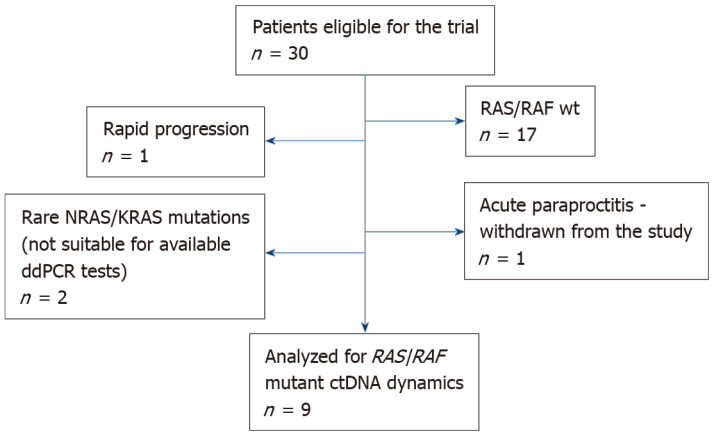


Figure 1 The flowchart of patients screening.

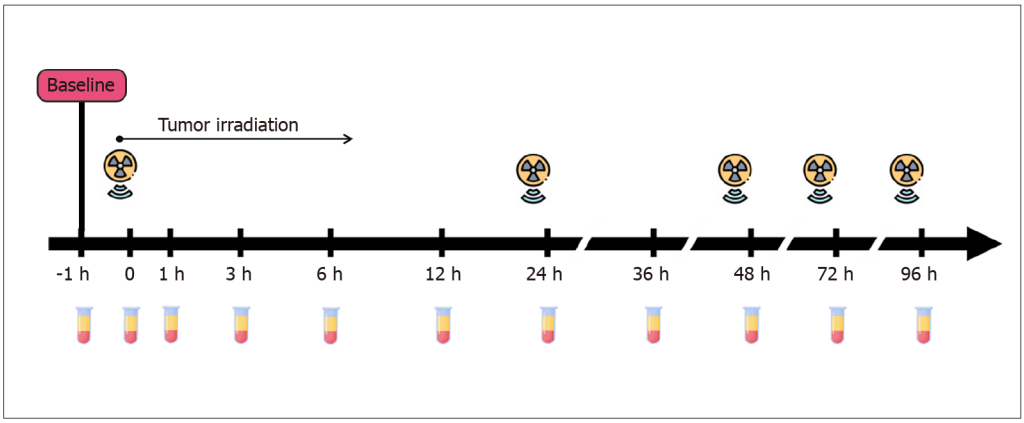


Figure 2 Schedule for serial blood-takes and irradiation fractions.

Diefenbach *et al*[22]. Isolated DNA was subsequently diluted in sterile distilled water and frozen at −24 °C until further analysis.

ctDNA analysis

The fractions of *KRAS/NRAS* mutations in codons 12, 13, 61 or *BRAF V600E* allele were measured by ddPCR using the QX100 Bio-Rad System[23]. ddPCR reactions were performed in triplicate. These reactions contained 2X ddPCR Supermix for Probes (no UTP, Bio-Rad), mutation-specific oligonucleotides (see Supplementary Tables 3 and 4) and 2-3 μL of the template DNA in a total reaction volume of 22-23 μL. Data analyses were performed with the QuantaSoft Software version 1.7.4 as recommended by the manufacturer. All ddPCR reactions, which yielded 10 or more droplets with the target DNA molecule, were considered informative. The absolute number of tumor-derived "mutated" DNA copies in 1 mL of plasma (C_{mut}) was calculated according to the formula:

$$\text{N mut copies/1 mL plasma} = \frac{\text{Concentration}\left(\frac{\text{copies}}{\mu\text{L}}\text{cfDNA}\right) \times \text{V templat}}{\text{V plasma}}$$

Where: Concentration – number of «mutated» droplets per 1 μL of ddPCR reaction; $V_{template}$ – volume of ctDNA aliquot taken into ddPCR, μL; $V_{dilution}$ – total volume of diluted ctDNA sample collected from the plasma, μL; V_{plasma} – volume of processed plasma, mL.

Statistics

The change of ctDNA content after tumor irradiation was evaluated according to the following formula:

$$\text{Percentage change (\%)} = 100\% \times \frac{\text{final Cmut} - \text{initial Cmut}}{\text{initial Cmut}}$$

Quantitative data were present as a median values/range or means ± 95% confidence interval (1.960σx). The non-parametric Wilcoxon Signed Rank Test and Mann–Whitney *U* test were utilized to compare the medians. *P* value of < 0.05 was considered statistically significant. All calculations were performed using IBM SPSS v.23 software package.

RESULTS

Nine rectal patients were included in the study of ctDNA fluctuations occurring within the first hours after RT. Individual characteristics of the patients are given in Table 1. Four out of nine (44%) analyzed subjects had detectable *RAS*/*RAF* mutations in plasma DNA at baseline (at least 5 mutation-specific signals per reaction). The probability of detecting ctDNA in plasma did not correlate with any clinical characteristics, *e.g.* age, gender, mutation type, T/N stages, tumor grade, tumor location within the rectum, extramural venous invasion, circumferential resection margin, tumor response to treatment or PFS (statistical data not shown).

Three of 5 patients, who were negative for plasma *RAS*/*RAF*-mutated DNA at baseline, did not show the presence of mutated copies in subsequent serial samples obtained after tumor irradiation. The remaining two patients (#ArAS and #GaZM) demonstrated an appearance of the mutated DNA copies within the follow-up period (Table 2 and Figure 3).

Four patients were recognized as "plasma-positive" at baseline. The mean concentration of mutated copies in plasma samples was 82 copies per 1 mL (22, 23, 39, and 244, respectively). The variant allele frequency (VAF) of circulating mutations ranged from 0.5% to 27.2%. The analysis of changes in mutated ctDNA concentration occurring within the first 96 h of treatment revealed a pronounced increase in the number of circulating *RAS*/*RAF* mutant copies in patients #DaKS, #ArTP, #MaNK (with the maximum percent changes equal to 509%, 174% and 71%, respectively). Patient #MaLI showed less consistent variations in ctDNA content, with a maximal concentration at the start (23 mutant copies per 1 mL plasma, VAF 2.3%) and a number of subsequent spikes and drops (Figure 3).

There was no correlation between the content of mutated ctDNA and the total irradiation dose accumulated during the blood collection time (r = -0.400; P (2-tailed) = 0.253, Spearman's Rho).

DISCUSSION

Patients with locally advanced rectal cancer provide a good opportunity for the analysis of RT-induced changes in the ctDNA level, as these malignancies frequently contain *RAS*/*RAF* mutations and the tumor irradiation is a part of routine clinical management of this disease[21]. The data obtained within this study are consistent with prior investigations, which were performed on lung cancer patients and demonstrated that radiotherapeutic or chemoradiotherapeutic intervention may result in a transient increase of the level of ctDNA in some cases[14-16]. As compared to published reports[14-16], our study considered multiple evenly distributed time points within the first day after tumor irradiation. We anticipated, that this additional effort may help us to identify a time interval characterized by maximal RT-induced ctDNA release. However, there was a significant interpatient variability with regard to the

Table 1 Patients, tumors and treatment characteristics

Patient ID	Gender	Age	cT	cN	Stage	CRM	EMV	Tumor location	RAS/RAFstatus	Total RT dose, Gy	Chemosensibilization[1]	RECIST	Surgery	MRI TRG	Mandard TRG	ypT	yN	Progression[2]	Follow-up, mo	ctDNA positive at baseline
																		Yes/no		
ArAS	M	44	3	1	3	No	No	L	KRAS G12S	50	Yes	SD	Yes	III	NA	3	0	No	6.90	No
GaZM	F	66	3	2	3	Yes	No	U-M	KRAS G13D	46	Yes	PR	Yes	IV	4	4	1	No	4.03	No
DaKS	M	73	4	1	3	Yes	No	L	KRAS G12A	25 (short course)	No	PD	No	IV	NA	NA	NA	Yes	4.73	Yes
ArTP	F	81	4	1	3	Yes	Yes	L	KRAS Q61L	50	No	SD	Yes	III	3	3	0	Nd	5.80	Yes
MaLI	F	78	3	1	3	No	No	L	NRAS G12D	25(short course)	No	PD	No	IV	NA	NA	NA	Yes	3.97	Yes
MaNK	F	48	3	2	3	No	Yes	M	KRAS G12D	50	Yes	SD	Yes	III	2	3	2	No	6.57	Yes
ZuNM	F	63	3	2	3	No	No	L-M	BRAF V600E	44	Yes	PR	Yes	II	2	3	0	No	5.13	No
MiMF	F	74	3	1	3	No	Yes	M	NRAS G12D	25(short course)	No	PR	No	III	NA	NA	NA	No	3.13	No
SaVV	M	65	3	1	3	Yes	No	L-M	NRAS Q61R	50	Yes	SD	No	III	NA	NA	NA	Nd	1.13	No

[1]Capecitabine 825 mg/m^2 twice daily was delivered on the days of RT (D1-5, D8-12, D 15-19, D22-26, D29-33).

[2]Disease status at March 1,.2021; Nd – lost from follow-up.

Tumor localization: U: Upper rectum, M: Middle rectum, L: Lower rectum; EMV: Extramural venous invasion; CRM: Circumferential resection margin; TRG: Tumor regression grade; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; NA: Not evaluated; ctDNA: Circulating tumor DNA.

timing of ctDNA concentration peaks (Table 2 and Figure 3).

There are several limitations of this investigation. Human studies involving multiple serial blood takes are logistically complicated and need to be well balanced with ethical issues, therefore it is understandable that our study and similar reports [14-16] are of limited size. Furthermore, the range of "natural" variations of ctDNA measurements occurring due to imperfect reproducibility of laboratory protocols or physiological fluctuations of ctDNA content is largely unknown. Therefore, although our study demonstrated a trend towards the RT-induced increase of ctDNA concentration in some rectal cancer patients, it is not clear how these observations need to be adjusted for the described above confounding factors. This limitation is also applicable to other published data sets[14-16].

The analysis of tumor-specific mutations at the initial diagnostic work-up is usually not complicated, given that the management of cancer patients always requires morphological visualization of transformed cells and thus implies the availability of malignant tissue. However, the detection of actionable mutations acquired during the

Table 2 Changes in circulating tumor DNA content during radiotherapy for locally advanced rectal cancer

Patient ID	Mutation	Baseline mutated ctDNA	ctDNA analysis	-1 h	0	1 h	3 h	6 h	12 h	24 h	36 h	48 h	72 h	96 h	Radiation dose per day / number of fractions / total dose accumulated for the blood collection time
ArAS	KRAS G12S	Neg	C (mut)[1]	0	0	0	3	0	0	0	0	14	12	nd	2.0 Gy/4 fr/8 Gy + capecitabine[3]
			C (mut + wt)[2]	642	1006	1337	1963	633	906	829	762	4298	3088	nd	
			VAF, %	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.4	nd	
GaZM	KRAS G13D	Neg	C (mut)	0	0	0	26	37	59	40	50	46	5	80	2.0 Gy/5 fr/10 Gy + capecitabine[3]
			C (mut + wt)	6	7	8	866	808	1450	604	441	506	112	1219	
			VAF, %	0.0	0.0	0.0	3.0	4.6	4.1	6.6	11.3	9.1	4.5	6.6	
DaKS	KRAS G12A	Pos	C(mut)	22	22	52	134	42	33	32	102	27	65	36	5.0 Gy/5fr/25 Gy
			C (mut + wt)	295	118	655	1802	588	292	170	549	359	391	331	
			VAF, %	7.5	18.6	7.9	7.4	7.1	11.3	18.8	18.6	7.5	16.6	10.9	
ArTP	KRAS Q61L	Pos	C (mut)	39	0	0	26	10	0	39	nd	42	107	nd	2.0 Gy/4 fr/8 Gy
			C (mut + wt)	7606	9430	4036	5028	3187	3033	12574	nd	10622	14443	nd	
			VAF, %	0.5	0.0	0.0	0.5	0.3	0.0	0.3	nd	0.4	0.7	nd	
MaLI	NRAS G12D	Pos	C (mut)	23	15	0	0	0	5	13	15	10	21	15	5.0 Gy/5 fr/25 Gy
			C (mut + wt)	981	1011	1073	1303	1309	1160	1010	724	815	673	1088	
			VAF, %	2.3	1.5	0.0	0.0	0.0	0.0	1.3	2.1	1.2	3.1	1.4	
MaNK	KRAS G12D	Pos	C (mut)	244	257	nd	nd	nd	Nd	254	335	415	418	387	2.0 Gy/5 fr/10 Gy + capecitabine[3]
			C (mut + wt)	897	1091	nd	nd	nd	Nd	1424	1832	2241	1719	1955	
			VAF, %	27.2	23.6	nd	nd	nd	Nd	17.8	18.0	18.5	24.3	19.8	
ZuNM	BRAF V600E	Neg	C (mut)	0	0	0	0	0	0	0	0	0	0	0	2.0 Gy/5 fr/10 Gy + capecitabine[3]
			C (mut + wt)	1652	372	522	522	522	522	671	837	1004	791	2805	
			VAF, %	0	0	0	0	0	0	0	0	0	0	0	
MiMF	NRAS G12D	Neg	C (mut)	0	0	0	0	0	0	0	0	0	0	0	5.0 Gy/5 fr/25 Gy
			C (mut + wt)	594	500	740	2294	804	681	681	386	644	2891	1536	
			VAF, %	0	0	0	0	0	0	0	0	0	0	0	
SaVV	NRAS Q61R	Neg	C (mut)	0	0	0	0	0	0	0	0	0	0	0	2.0 Gy/5 fr/10 Gy + capecitabine[3]

C (mut + wt)	196	237	541	840	621	748	353	372	272	261	249
VAF, %	0	0	0	0	0	0	0	0	0	0	0

[1]C (mut): Number of mutated copies per 1 mL of plasma measured by droplet digital PCR.
[2]C (mut + wt): Total number of target fragment (both *wt* and *mut*) copies per 1 mL of plasma measured by droplet digital PCR.
[3]Capecitabine 825 mg/m^2 twice daily was delivered on the 1-5 d of RT.
ctDNA: Circulating tumor DNA; VAF: Variant allele frequency.

course of therapy presents a challenge. For example, the management of lung cancer patients, whose tumors progressed during gefitinib, erlotinib or afatinib treatment, involves the analysis of EGFR T790M mutation. The presence of this mutation justifies the administration of osimertinib, while the absence of this substitution calls for other treatment options. Re-biopsy of multiple visceral tumor lumps is often not feasible; therefore, the analysis of EGFR T790M mutation usually relies on liquid biopsy[24,25]. Clinical studies demonstrate that the detection of EGFR T790M mutation in plasma is seriously compromised by the low sensitivity of the test, especially in patients with limited tumor burden[26,27].

This study utilized patients with localized rectal cancer, who had a moderate volume of tumor masses. It is therefore explainable that only 4 out of 9 patients had detectable ctDNA at baseline. These data are comparable with the results obtained in other studies[12,28]. We deliberately focused on rectal cancer disease, as these patients often receive irradiation during the standard preoperative treatment, so no additional interventions were involved within this investigation. We demonstrated that two out of five subjects, who were initially ctDNA-negative, showed the presence of mutated DNA copies in the plasma after the start of the therapy. In addition, 3 out of 4 initially ctDNA-positive subjects experienced a RT-related increase of ctDNA content. The obtained data look promising, so further studies, which involve tumor irradiation not as a part of regular treatment plan, but as an additional intervention aimed to support ctDNA analysis, appear to be justified.

The clinical utility of this approach deserves to be evaluated in lung cancer patients, who demonstrate the disease progression during the therapy by first- or second-generation EGFR inhibitors and therefore require the diagnostic detection of EGFR T790M substitution. It is feasible to organize a prospective study, where the tumor lumps observed in these patients will be subjected to irradiation in order to provoke the release of tumor DNA in the bloodstream. It is essential to minimize the risks of this procedure by considering the anatomic location of targeted tumor foci (particularly, the vicinity of large blood vessels), ensuring a highly precise topical delivery of the irradiation dose and accounting for potentially significant comorbidities. If this intervention was to increase the rate of EGFR T790M allele detection in the plasma while being sufficiently safe, the proposed approach would have significant potential for clinical use.

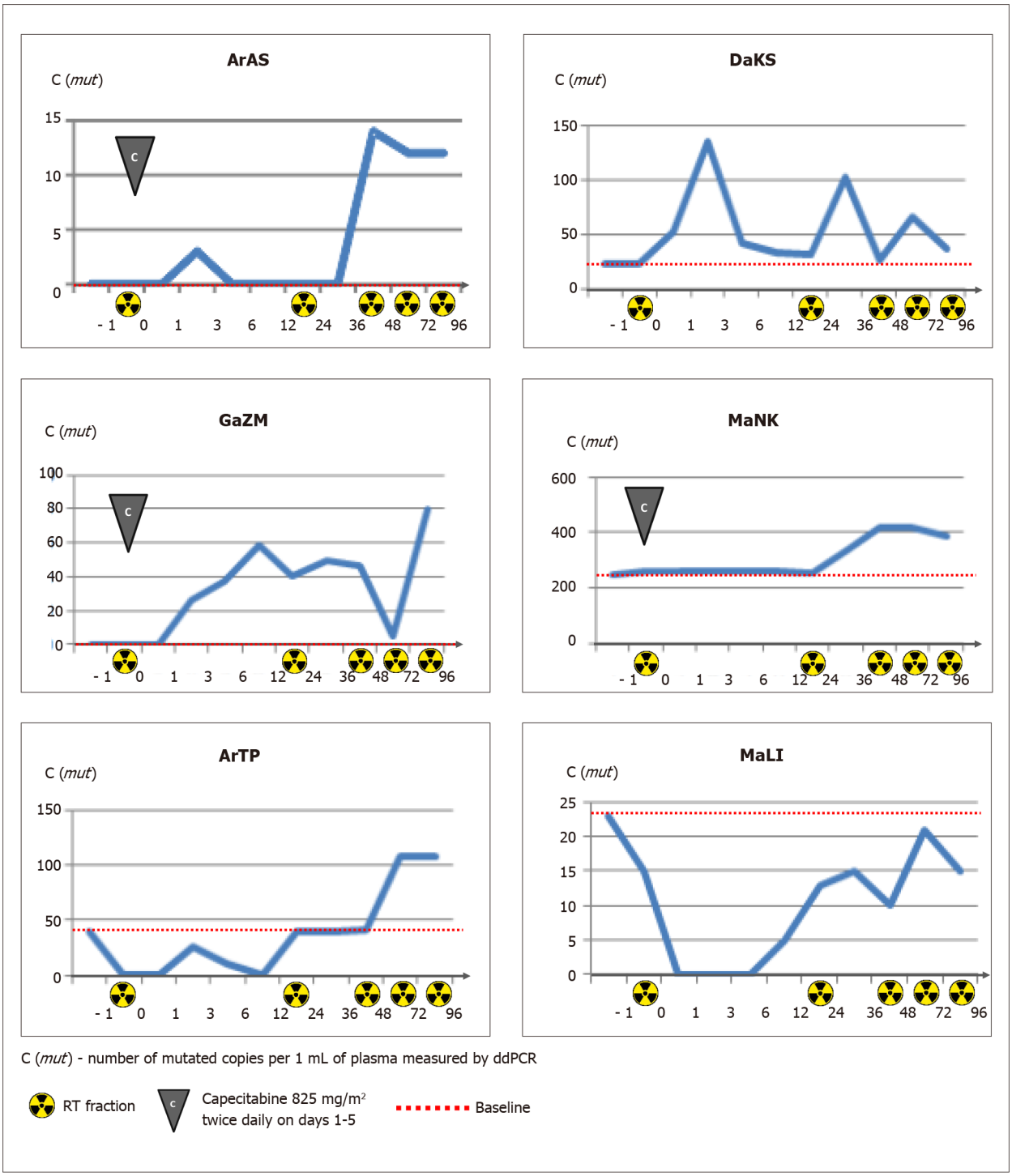


Figure 3 Changes in ctDNA content occurring within first 96 h after the start of radiotherapy.

CONCLUSION

Local tumor irradiation may facilitate the detection of plasma ctDNA. This study calls for a comprehensive evaluation of the clinical feasibility of irradiation-assisted liquid biopsy.

ARTICLE HIGHLIGHTS

Research background

The detection of circulating tumor DNA (ctDNA) is a valuable diagnostic tool,

however many cancer patients do not have detectable amount of ctDNA in their plasma.

Research motivation

We evaluated whether tumor irradiation may provoke the release of tumor DNA in the bloodstream and thus improve the efficiency of liquid biopsy.

Research objectives

We have chosen for the study patients with locally advanced rectal cancer as they usually receive preoperative tumor irradiation as a part of standard treatment plan.

Research methods

The study included 9 patients with *RAF/RAF* mutations. Multiple serial blood draws were taken within first 96 h after the first fraction of radiotherapy. The amount of mutated *RAF/RAF* copies in the plasma was quantified by the droplet digital PCR.

Research results

Five out of nine patients demonstrated increased ctDNA content at least at some plasma samples obtained after the beginning of radiotherapy.

Research conclusions

Radiotherapy is a promising tool for the improvement of performance of liquid biopsy.

Research perspectives

It is feasible to extend this study to lung cancer patients, who receive tyrosine kinase inhibitors and may experience acquired tumor resistance due to the gain of secondary mutation.

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SYSTEMATIC REVIEWS

Mixed odontogenic tumors: A review of the clinicopathological and molecular features and changes in the WHO classification

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Abstract

BACKGROUND

Ameloblastic fibromas and ameloblastic fibrosarcomas are rare odontogenic tumors, and controversy exists in the classification of cases presenting hard-tissue production: Ameloblastic fibrodentinoma (AFD) and ameloblastic fibro-odontoma (AFO). These cases are currently considered "developing odontomas" (hamartomatous lesions).

AIM

To analyze the clinicopathologic features of these lesions and discuss the changes in the 2017 World Health Organization classification.

METHODS

An electronic literature search was performed in the PubMed/MEDLINE database. An electronic search of the English language literature was performed and last updated in September 2020 in the PubMed/MEDLINE database using the following terms: "ameloblastic fibroma", "ameloblastic fibrodentinoma", "ameloblastic fibro-odontoma", "ameloblastic sarcoma", "ameloblastic fibrosarcoma", "ameloblastic fibrodentinosarcoma", "ameloblastic fibroodontosarcoma" and "odontogenic carcinosarcoma". The inclusion criteria were odontogenic tumor series, case reports and systematic reviews that provided sufficient clinical, radiological and microscopic documentation to confirm the diagnosis.

RESULTS

The database search strategy resulted in 947 papers. Articles focusing on other

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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topics, articles that were not in English, duplicate articles, and articles without fulfilling the inclusion criteria were excluded. Finally, 96 publications were included in this review to describe and discuss the main features of the searched entities. Several aspects of AFO and AFD, such as biological behavior, age of occurrence, amount of hard tissue, and potential for malignant transformation into odontogenic sarcomas, support the neoplastic nature in most of the reported cases. Considering the clinical, radiographic, histopathological and molecular characteristics of odontogenic lesions with hard tissue production, we suggest that these types of lesions should continue to be recognized as odontogenic tumors by maintaining the classically used terms.

CONCLUSION
This recommendation will be relevant for future clinical, microscopic, and molecular studies to better understand the biology of these interesting odontogenic tumors.

Key Words: Ameloblastic fibroma; Ameloblastic fibrosarcoma; Odontogenic carcinosarcoma; Odontogenic tumors

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Core Tip: We consider that the recent 2017 World Health Organization classification does not clarify the subject when considering ameloblastic fibrodentinoma (AFD) and ameloblastic fibro-odontoma (AFO) as "developing odontomas". According to the clinical, radiographical, histopathological and molecular features of the cases reviewed, we suggest that AFD and AFO should continue to be considered benign neoplasms. Thus, the nomenclature of these mixed benign odontogenic tumors would be congruent with the classification of ameloblastic/odontogenic sarcomas. Additionally, further studies are warranted to compare these interesting odontogenic tumors and finally better clarify and understand their similarities and differences.

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INTRODUCTION

Ameloblastic fibroma (AF) is a rare, benign odontogenic tumor formed by odontogenic ectomesenchyme that resembles the dental papilla, has embedded epithelial strands and nests, and is similar to dental lamina and enamel organs but without the presence of hard tissues. If the lesion has dentinoid material, it must be denominated as ameloblastic fibrodentinoma (AFD); when it produces enamel/enamel matrix, it is known as ameloblastic fibro-odontoma (AFO), independent of the amount of hard tissue present[1]. In 2005, WHO classified AF and AFD together, making no distinctions regarding epidemiological and clinical features. Microscopically, the only difference between AF and AFD is the presence of dentinoid in the latter. AFO affects younger patients and has shown a better prognosis than AF/AFD[1]. However, the WHO classification of 2017 states that AF rarely produces dental hard tissues and cases formerly considered AFD/AFO rather represent developing odontomas[2].

This group of mixed odontogenic tumors (AF and related-lesions) histologically resembles different tooth formation stages, particularly when dentin and enamel are produced, sharing similar morphologic features with the so-called "developing odontoma", which is considered a tumor-like malformation or hamartoma by WHO. Nevertheless, unlike odontomas, these mixed tumors present characteristics that support the concept of a true neoplasm, such as biological behavior, age of occurrence, and well-documented cases of malignant transformation into odontogenic sarcomas, namely, ameloblastic fibrosarcoma (AFS), ameloblastic fibrodentinosarcoma (AFDS)

and ameloblastic fibro-odontosarcoma (AFOS). Moreover, the recent publication of a few reports of odontogenic carcinosarcomas led to its inclusion as a specific tumor by WHO in 2017[2-4].

This review is based on the WHO classification of 2005, because most of the literature is based on this nomenclature, and it was performed to analyze the clinicopathologic features of these lesions to discuss the changes in the 2017 WHO classification.

MATERIALS AND METHODS

An electronic search of the English language literature was performed and last updated in September 2020 in the PubMed/MEDLINE database using the following terms: "ameloblastic fibroma", "ameloblastic fibrodentinoma", "ameloblastic fibro-odontoma", "ameloblastic sarcoma", "ameloblastic fibrosarcoma", "ameloblastic fibrodentinosarcoma", "ameloblastic fibroodontosarcoma" and "odontogenic carcinosarcoma".

Previous cases that did not use the current terminology for these tumors, recently identified as AF, AFD, AFO, AFS, AFDS or AFOS, were also found and evaluated for possible inclusion.

The inclusion criteria were odontogenic tumor series, case reports and systematic reviews including AF, AFD, AFO, AFS, AFDS or AFOS, which provided sufficient clinical, radiological and microscopic documentation to confirm the diagnosis. Reports without this information were excluded.

RESULTS

The database search strategy resulted in 947 papers. Articles focusing on other topics, articles that were not in English, duplicate articles, and articles without fulfilling the inclusion criteria were excluded. Finally, 96 publications were included in this review to describe and discuss the main features of the searched entities.

AF

Clinical characteristics: This uncommon benign mixed odontogenic tumor occurs preferentially in children and young adults, with a mean age of 14.9 years, ranging from 7 wk to 57 years. Only 20% of the cases are diagnosed in patients older than 20 years. Considering all odontogenic tumors, AF represents only 0.6% to 3.1% of these neoplasms. Most of the cases affect the mandible, with a slight predilection for male patients, with a male/female ratio of 1.4:1. The size of AF when diagnosed varies from 0.7 to 16 cm (mean of 4.05 cm)[5].

Most cases present as painless jaw swelling or are discovered during routine radiographical examination due to delayed tooth eruption, eventually causing cortical expansion and facial asymmetry. Approximately 80% of AF involves the posterior region of the mandible but has also been found on the posterior maxilla and rarely in the anterior region of the jaws[5,6].

Radiographic features: Radiographically, AF presents as a well-defined, unilocular (56%) or multilocular (44%) radiolucent lesion, with regular and well-defined margins, typically sclerotic (94%). Tumors measuring less than 5 cm usually tend to be unilocular. Approximately 80% of cases are associated with a single or several unerupted teeth, usually of permanent dentition. Root resorption and cortical perforation are uncommon and described in 8.1% and 5.2% of cases, respectively[5,6].

Histopathology: Microscopically, AF is a mixed tumor with variable amounts of epithelial and ectomesenchymal components in different areas of the same lesion. The ectomesenchyme resembles the embryonic dental papilla, comprising a myxoid cell-rich stroma involving odontogenic epithelial elements that may present different patterns: epithelial strands, comprising a double layer of cuboidal cells (Figure 1A); cords with tooth bud-like projections of cuboidal cells (Figure 1B); epithelial follicles comprising a layer of peripheral tall columnar ameloblast-like cells and a central area, displaying more loosely arranged stellate/spindle-shaped cells, similar to the stellate reticulum of the enamel organ (Figure 1C); clefts of mesenchymal tissue surrounding follicular epithelial proliferation can be present (Figure 1D); and smaller epithelial rosette-like islands that resemble remnants of dental lamina may be observed

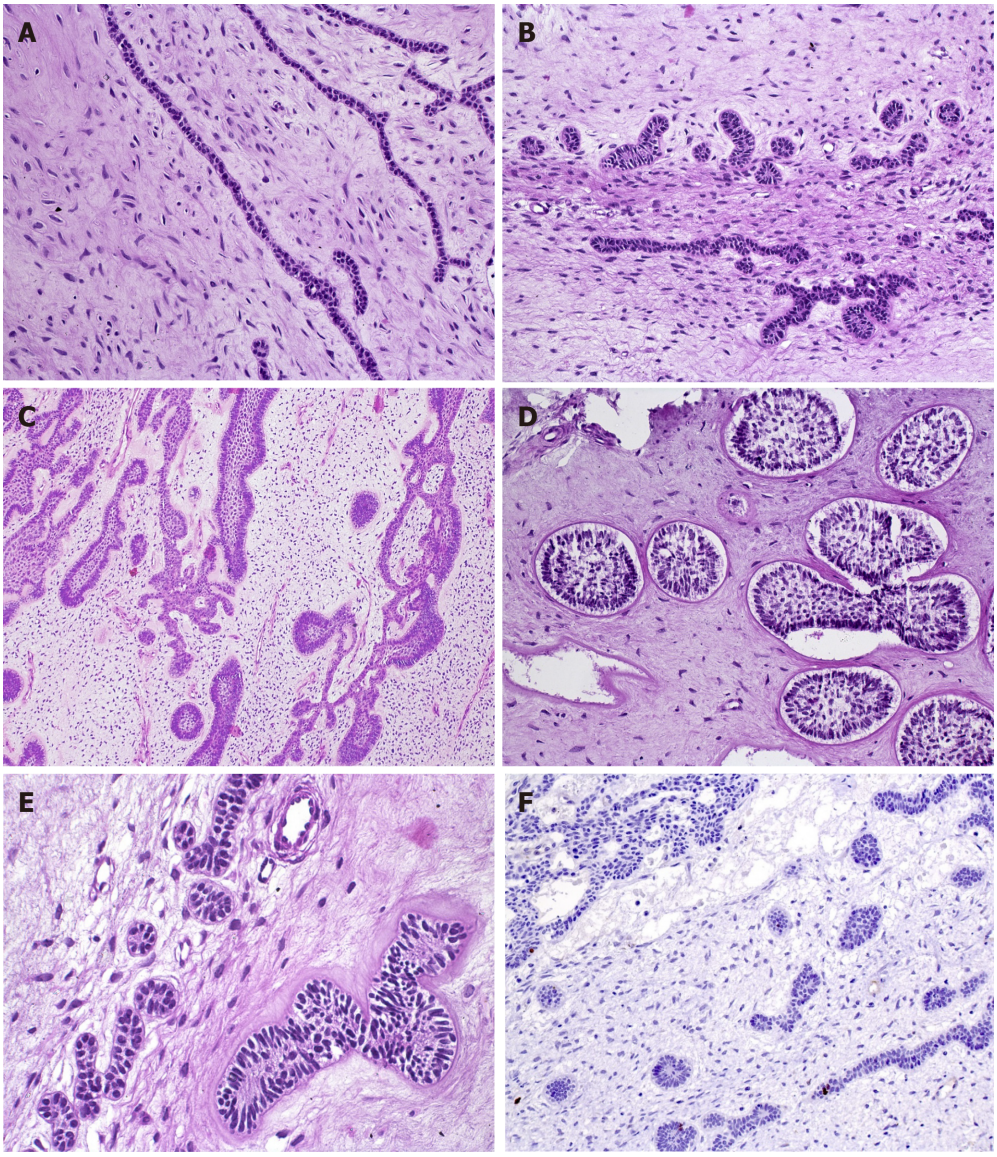


Figure 1 Diverse aspects of the odontogenic epithelium in ameloblastic fibromas within the cell-rich myxoid stroma. A: Epithelial strands, comprising a double layer of cuboidal cells (HE, 20×); B: Epithelial proliferation with primitive appearance that resembles tooth bud-like structures (HE, 20×); C: Epithelial component with a follicular pattern comprising columnar cells at the periphery of the nests with central stellate reticulum-like cells (HE, 10×); D: Clefts of mesenchymal tissue surrounding follicular epithelial proliferations (HE, 20×); E: Mild hyalinization surrounding the basal layer of the epithelial nest (left). Smaller epithelial rosette-like islands resemble remnants of dental lamina (right) (HE, 40×); F: A very low rate of proliferation in both mesenchymal and epithelial components, showing the benign behavior of ameloblastic fibromas (IHC for Ki-67, 20×).

(Figure 1E). Mitotic figures in either the epithelium or mesenchyme are uncommon, a finding consistent with the benign nature of the tumor. According to the 2005 WHO classification, no hard tissues, such as enamel or dentin, are present[1]. In the 2017 classification, AF rarely presented dental hard tissue formation that eventually reached an exceptional size[2]. According to the histopathological criteria of 2005, 280 cases of AF were identified in the literature. The proliferation rate is low, with a Ki-67 Labeling index generally lower than 3% (Figure 1F)[5-7].

Treatment and prognosis: Most reported AFs were treated conservatively by enucleation and curettage. Radical surgery is used in more extensive tumors or recurrent lesions. Recurrence was reported in 16.3% of cases, and malignant transformation into AFS was cited in 6.4%. Recurrence seems to be more common in younger patients and malignant transformation more common in older patients[5].

AFD

Clinical characteristics: AFD is a rare benign odontogenic tumor with histopathological features of AF and the formation of dysplastic dentin. The WHO classification

of 2005 describes AF and AFD together, without further considerations of the latter, beyond the presence of dentin/dentinoid. No strong evidence of differences in the biological behavior of AF and AFD is available[1,8]. However, the 2017 WHO classification of tumors cited that lesions referred to as AFDs are more likely "developing odontomas", and the editors suggest that they are no longer being considered mixed odontogenic tumors, as in the previous classification[2].

AFD is rare, corresponding to less than 1% of all odontogenic tumors in most reported series. It usually presents as asymptomatic swelling, more frequently at the posterior mandible (mandible/maxilla ratio of 2.4:1), often associated with a permanent unerupted tooth. When a deciduous tooth is involved, the lesions are generally located in the incisor area. From the 45 cases reviewed, we found a slight male predilection, corresponding to 59.5% of cases, usually in the first and second decades of life; however, 17 of 45 (37.7%) cases occurred in the third decade and beyond. The mean age was 17.8 years, with an age range of 1 to 63 years (Table 1).

Radiographic features: Radiographically, AFD presents as a well-defined radiolucency with varying degrees of radiopacity, depending on the amount of calcified dentinoid. In 2012, Giraddi and Garg[9] reported a large and aggressive AFD with irregular borders, with considerable expansion and perforation of the cortical bone; however, the possibility of eventual foci of malignant transformation to AFDS should be considered in this case.

Histopathology: Microscopically, AFD is formed by odontogenic epithelium and ectomesenchyme arranged in an indistinguishable pattern from AF, in addition to the presence of dentinoid (Figure 2A, B). The epithelial cords and islands resemble the dental lamina and enamel organ, lying in myxoid cell-rich ectomesenchymal tissue with stellate-shaped fibroblasts resembling dental papilla. The amount of dentinoid material is variable, but minimal evidence is sufficient for the diagnosis to be accepted [1]. We found only 45 cases of AFD in the English literature according to these characteristics. Similar to AF, the Ki-67 index in AFD is low in both epithelial and mesenchymal components[8].

Treatment and prognosis: The treatment of choice is surgical, with enucleation of the lesion and unerupted tooth involvement. Recurrence is uncommon (9%) and likely a consequence of incomplete surgical removal. Radical surgery has been used in aggressive, atypical or recurrent lesions[9]. AFD rarely progresses into ameloblastic fibrodentinosarcoma, in which only the mesenchymal component shows malignant transformation. Only 4 cases of AFDS with a preexisting benign lesion have been described in the English literature (Table 2).

AFO

Clinical characteristics: AFO is a slow-growing, expansive, benign mixed odontogenic tumor that is histologically similar to AFD but also contains enameloid material in variable amounts[1]. Similar to AFD, the term AFO was excluded from the latest WHO classification, in which lesions with these characteristics are considered developing odontomas[2].

According to the literature, AFO occurs mainly in children, with a mean age of 9.6 years. It has a male predilection, with a ratio of 1.85:1 and an average size of 3.3 cm, ranging from 0.8 to 14 cm. More than 80% of cases affect the posterior portion of the mandible, eventually causing facial asymmetry[10,11]. To our knowledge, 222 cases have been reported in the English literature, among which 211 were reviewed by Chrcanovic *et al*[5] and 11 additional cases were published later[12-22], including one peripheral case[23]. One case was associated with paresthesia of the chin and lower lip in a 12-year-old girl[22].

Radiographic features: AFO usually appears as a well-defined unilocular mixed radiolucent-radiopaque lesion, frequently in close association with the crown of an unerupted tooth. It commonly causes painless cortical expansion but no perforation [10,11].

We reviewed the literature and found 82 cases with optimal radiographic documentation, among which 22 (26.8%) presented radiographically as a single large opaque mass similar to odontoma and 11 (13.4%) presented several foci of opacities; however, most cases were poor in hard tissues, with 43 cases presenting few opacities (52.4%) and 6 cases appearing as radiolucent lesions (7.3%).

Histopathology: Similar to AF, AFO comprises odontogenic epithelium and ectomesenchyme, but it also contains hard dental tissues in variable amounts and degrees of

Table 1 Reported 45 cases of ameloblastic fibrodentinoma found in the English language literature

Case	Ref.	Year	Sex/age	Location
1	Straith[42]	1936	F/30	Posterior mandible
2	Field and Ackerman[43]	1942	NA/9	Posterior mandible
3	Stafne[44]	1943	F/25	Posterior mandible
4	Stafne[44]	1943	NA/23	Posterior mandible
5	Stafne[45]	1946	NA	NA
6	Thoma and Goldman[46]	1946	M/6	Maxillary sinus area
7	Ingham[47]	1952	F/19	Posterior mandible
8	Sirsat[48]	1952	M/36	Maxillary sinus area
9	Husted and Pindborg[49]	1953	M/4	Anterior maxilla
10	Husted and Pindborg[49]	1953	F/63	Posterior mandible
11	Hitchin and White[50]	1955	M/4	Anterior mandible
12	Pindborg[51]	1955	M/20	Posterior mandible
13	Gorlin *et al*[52]	1961	F/4	Anterior maxilla
14	Gorlin *et al*[52]	1961	F/13	Posterior mandible
15	Gorlin *et al*[52]	1961	M/8	Posterior mandible
16	Azaz *et al*[53]	1967	M/4.5	Anterior mandible
17	Manning and Browne[54]	1970	F/55	Posterior mandible
18	Hoggins and Browne[55]	1976	M/24	Posterior mandible
19	Gulmen *et al*[56]	1976	M/30	Anterior mandible
20	Godjesk *et al*[57]	1980	M/3.5	Anterior mandible
21	Rennie and Critchlow[58]	1981	M/7	Posterior maxilla
22	van Wyk and van der Vyver[59]	1983	M/8	Posterior mandible
23	Villafañe *et al*[60]	1986	F/22	Posterior maxilla
24	Lukinmaa *et al*[61]	1897	M/11	Posterior mandible
25	Anker and Radden[62]	1989	F/24	Posterior mandible
26	Ulmansky *et al*[63]	1994	M/60	Posterior maxilla
27	Ulmansky *et al*[63]	1994	M/8	Posterior maxilla
28	Cassidy *et al*[64]	1987	M/12	Posterior maxilla
29	Akal *et al*[65]	1997	M/9	Posterior mandible
30	Akal *et al*[65]	1997	M/22	Mandible
31	Takeda *et al*[66]	2000	M/21	Mandibular retromolar area
32	Karasu *et al*[67]	2004	F/21	NA
33	Bhargava *et al*[68]	2011	M/51	Anterior maxilla
34	Giraddi and Garg[9]	2012	F/17	Mandible
35	Bologna-Molina *et al*[8]	2013	F/1.5	Mandible
36	Sankireddy *et al*[69]	2013	M/14	Anterior maxilla
37	Salehinejad *et al*[70]	2013	F/13	Anterior mandible
38	Ikeda *et al*[71]	2014	F/8	Posterior mandible
39	Lee *et al*[72][1]	2014	F/4	Anterior mandible
40	Unsal *et al*[73]	2014	M/11	Anterior mandible
41	Joseph *et al*[74]	2015	M/12	Anterior Maxilla

42	Costa *et al*[75]	2015	F/12	Posterior mandible
43	Bhargava *et al*[76]	2016	M/1	Anterior mandible
44	Bavle *et al*[77]	2017	F/14	Posterior mandible
45	Sabu *et al*[78]	2018	M/20	Mandible (left body to right parasymphysis)

[1]Associated with calcifying cystic odontogenic tumor, only dentinoid production.
F: Female; M: Male; NA: Not available.

Table 2 Main data of 21 cases reported of ameloblastic fibrodentinosarcoma/ameloblastic fibro-odontosarcoma in the literature

Case	Ref.	Sex/age	Location	Mineralized tissues	Preexisting tumor	Progression
1	Villa[79]	F/20 yr	Posterior mandible	Enamel	Yes (NA)	Recurrence
2	Forman and Garret[80]	M/17 yr	Posterior mandible	Dentin and enamel	No	No recurrence
3	Altini and Smith[81]	M/27 yr	Mandible	Dentin	No	NA
4	Takeda *et al*[32]	M/19 yr	Maxilla	Dentin	AF	Recurrence and death
5	Howell and Burkes[31]	F/18 yr	Posterior mandible	Dentin and enamel	AFO	Recurrence, metastasis and death
6	Howell and Burkes[31]	M/36 yr	Posterior mandible	Dentin	AFO	Recurrence
7	Altini *et al*[41]	M/25 yr	Mandible	Dentin	No	No recurrence
8	Takeda *et al*[33]	M/23 yr	Mandible	Dentin and enamel	No	Recurrence and death
9	Corominas-Villafañe *et al*[82]	M/12 yr	Mandible	NA	AF	No recurrence
10	Herzog *et al*[83][1]	F/14 yr	Mandible	NA	AFO	NA
11	Bregni *et al*[25]	M/32 yr	Mandible	Dentin	No	NA
12	Muller *et a*[84]	M/83 yr	Mandible	Dentin and enamel	AFO	Recurrence
13	Zabolinejad *et al*[35]	M/4 mo	Maxillary sinus	Dentin	No	No recurrence
14	Mainenti *et al*[34]	F/12 yr	Mandible	Dentin and enamel	AFO	No recurrence
15	Wang *et al*[30]	F/45 yr	Posterior mandible	Dentin and enamel	No	No recurrence
16	Reiser *et al*[85]	F/6 yr	Mandible	Dentin and enamel	No	No recurrence
17	Khan *et al*[86]	F/17 yr	Mandible	NA	No	NA
18	Gatz *et al et al*[87]	F/14 yr	Maxilla	Dentin	AFO	Recurrence
19	Chen *et al*[88]	M/4 yr	Mandible	Dentin and enamel	No	No recurrence
20	Niu *et al*[89]	F/31 yr	Mandible	Dentin and enamel	No	No recurrence at 3 months, lost follow-up
21	Atarbashi-Moghadam *et al*[90]	F/32 yr	Mandible	Dentin	No	Recurrence and metastasis

[1]Article in German, abstract in English.
F: Female; M: Male; AF: Ameloblastic fibroma; AFO: Ameloblastic fibro-odontoma; AFDS: Ameloblastic fibrodentinosarcoma; AFOS: Ameloblastic fibroodontosarcoma; NA: Not available.

maturation, such as enameloid and dentinoid (Figure 2C-F). Frequently, Ki-67 is lower than 1% in epithelial and mesenchymal cells[1,24].

Treatment and prognosis: The treatment of choice is conservative surgery through enucleation and curettage, with removal of the unerupted tooth. Recurrence is uncommon, and malignant transformation is very rare, with only 6 cases reported to

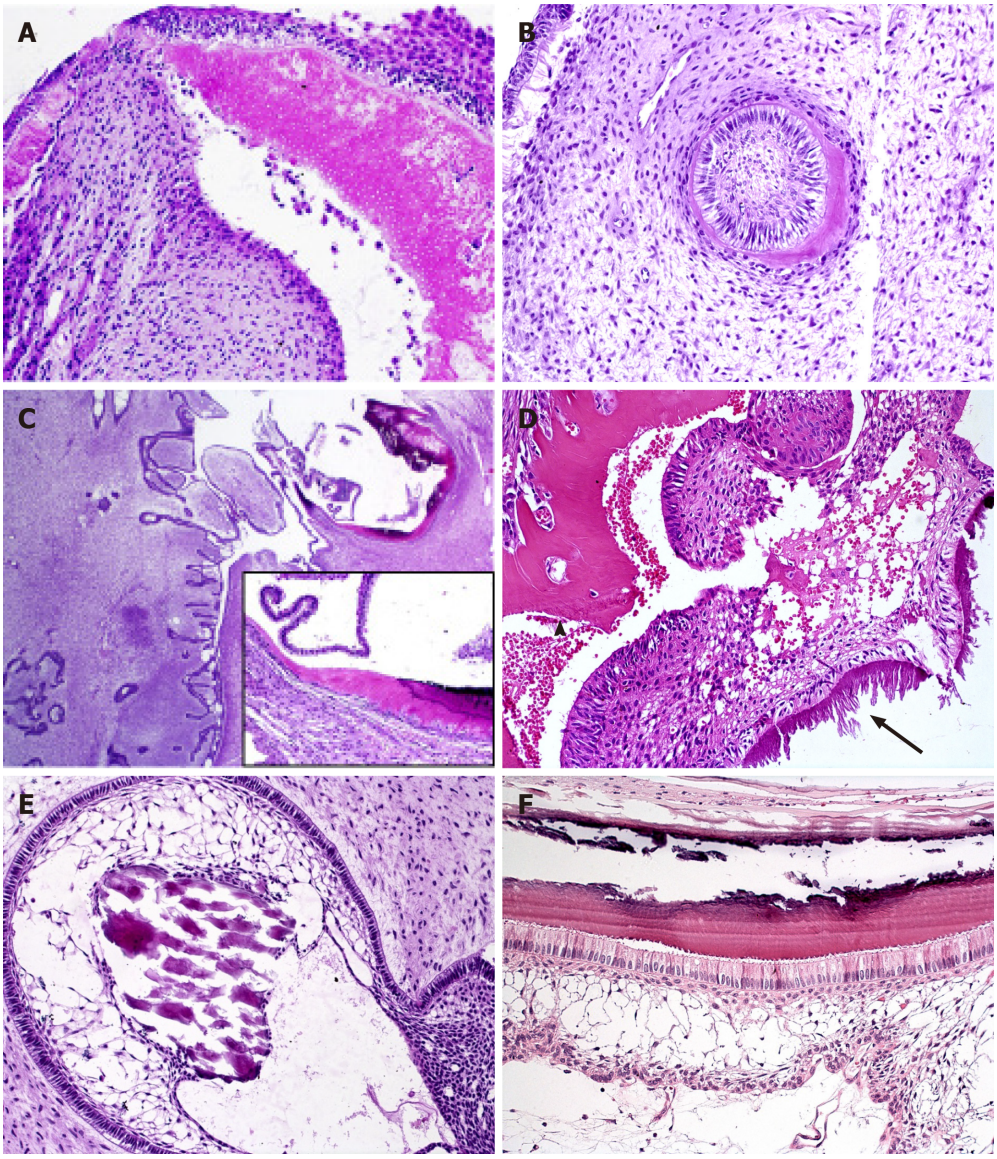


Figure 2 Mineralized tissue formation in ameloblastic fibrodentinoma and ameloblastic fibro-odontoma. A, B: Dentinoid induction by epithelial cells in ameloblastic fibrodentinoma; note the presence of tubules in (A) (HE, 20×); C: Prominent proliferation of soft tissue similar to ameloblastic fibromas and focal areas of dentinoid and enamel matrix production in close relationship with the epithelial component in ameloblastic fibro-odontoma (HE, 2.5×; inset 20×); D: Structures similar to tubules are observed in the dentinoid (arrowhead), which can be associated with odontogenic epithelium or ectomesenchymal tissue, while enamel matrix (arrow) associated with columnar odontogenic appears more basophilic, with different patterns of deposition that can resemble prisms or globules (HE, 20×); E: Calcificated material, compatible with enameloid, in direct relationship with epithelial cells of the stellate reticulum-like area (HE, 20×); F: Details of the columnar ameloblast-like cells with reverse polarization producing enamel matrix in which the "fish scale" pattern is visible. Flattened cells between the columnar cells and stellate reticulum-like area resemble the stratum intermedium of the tooth germ, which is believed to assist the ameloblast in producing enamel during odontogenesis (HE, 40×).

date[5,10].

AFS

Clinical characteristics: AFS is a very rare malignant odontogenic tumor, considered the malignant counterpart of AF, in which the ectomesenchymal component shows features of sarcoma, while the odontogenic epithelium remains bland[1]. To date, up to 100 cases of AFS have been reported in the English language literature. The mean age of the affected patients was 28 years (range: 3 to 89 years), with a slight predilection for male patients (male-to-female ratio: 1.6:1). AFS is more frequent in the mandible, and up to one-third of the cases have been derived from previously documented AF. Patients with AFS originating from AF have a mean age of 33 years, and those with *de novo* AFS (previous AF not demonstrated) are one decade younger. AFS usually appears as painful swelling, and paraesthesia may be present[5,25,26].

Radiographic features: As with most malignant intraosseous tumors, AFS presents as expansive ill-defined unilocular or multilocular radiolucency with bone destruction areas, perforation of cortical areas, irregular margins and occasional root resorption. It can be associated with unerupted teeth and eventually cause diffuse expansion and thinning of the cortex[2,27].

Histopathology: Histologically, AFS is similar to AF; however, the ectomesenchyme is hypercellular and malignant, while the epithelial component tends to decrease and virtually disappears in recurrent tumors (Figure 3A-C)[1,28]. Epithelial nests and cords remain inactive, presenting an immunohistochemical profile similar to AF and positivity for AE1/AE3[15-17] (Figure 3E). Proliferation-related markers such as Ki-67 and p53 can help distinguish AF and AFS, because they are virtually negative in the mesenchymal component and epithelium of AF and positive in a variable percentage in the malignant cells of AFS (Figure 3F).

Treatment and prognosis: Because AFS is locally very aggressive, with a high tendency to relapse, treatment includes wide surgical removal and long-term follow-up. Adjuvant radiotherapy has been used in some cases with favorable results[29], while the usefulness of chemotherapy has not been confirmed[26]. Recurrence occurs in approximately 20% and metastasis in only 4.5% of cases, but the mortality after 5 years of treatment is relatively high, estimated in 25.4% of cases[1,25,27].

Ameloblastic fibrodentinosarcoma/ameloblastic fibro-odontosarcoma

In the 2005 and 2017 WHO classifications of tumors, AFDS/AFOS are described together as tumors with histological features of AFS presenting dentinoid (fibrodentinosarcoma) or dentinoid and enameloid (fibro-odontosarcoma) (Figure 3D) [1,2].

Clinical and histopathological features: Clinically, AFDS/AFOS present as painful swelling of the jaws, with only 21 cases reported in the literature, as summarized in Table 2[9,30]. From these cases, 10 described enameloid formation, corresponding to AFOS. The age range of the reported cases was from 4 mo to 83 years, with a peak in the third decade. Approximately 40% of the cases recurred, two developed metastasis, and three patients died because of aggressive local invasion[31-33].

Immunohistochemically, AFDS/AFOS are similar to AFS, with odontogenic epithelium positive for AE1/AE3, facilitating the localization of epithelial nests in cases of mesenchymal predominance, excluding the diagnosis of other sarcomas. As discussed previously, proliferation markers such as Ki-67 and p53 confirm the aggressiveness of the lesion, helping to differentiate it from its respective benign counterpart [30,34,35].

Radiographic features: Radiographically, they appear as a uni- or multilocular radiolucency with variable dense opacities associated with impacted teeth. Irregular borders, expansion and perforation of the cortex are common, indicating a malignant tumor[34].

Treatment and prognosis: Treatment is based on wide local surgical excision, and long-term follow-up is advised[30]. We found 21 cases of AFDS/AFOS in the English language literature, 8 of which (38%) recurred, 2 developed distant metastasis, and 3 cases (15%) caused death.

Molecular characterization of mixed odontogenic tumors

To date, few studies have investigated the genetic/molecular profiling of mixed odontogenic tumors. Molecular testing (polymerase chain reaction followed by direct sequencing and next-generation sequencing) has revealed that 33% to 100% of benign mixed odontogenic tumors (AF, AFD, and AFO) and 71% of AFS harbor *BRAF* p.V600E mutation in their mesenchymal component (and rare cases in both components), unlike odontomas, which are BRAF wild-type. This finding suggests that a subset of AF, AFD and AFO differs molecularly from odontomas, likely supporting the distinct nature of these entities (neoplastic *vs* hamartomatous). The *BRAF* p.V600E mutation is involved in the pathogenesis of several tumors, including ameloblastoma, playing a role as a downstream activator of the MAPK signaling pathway, which regulates several cell processes, such as proliferation, survival and apoptosis[36-38].Confirming these findings, immunohistochemical reactions against BRAFV600E exhibited specific staining only in the stromal component, supporting the role of this mutation as a driver of the malignant stromal component[38]. Although the BRAF p.V600E mutation seems to be present in most AFSs in the study of Agaimy *et al*

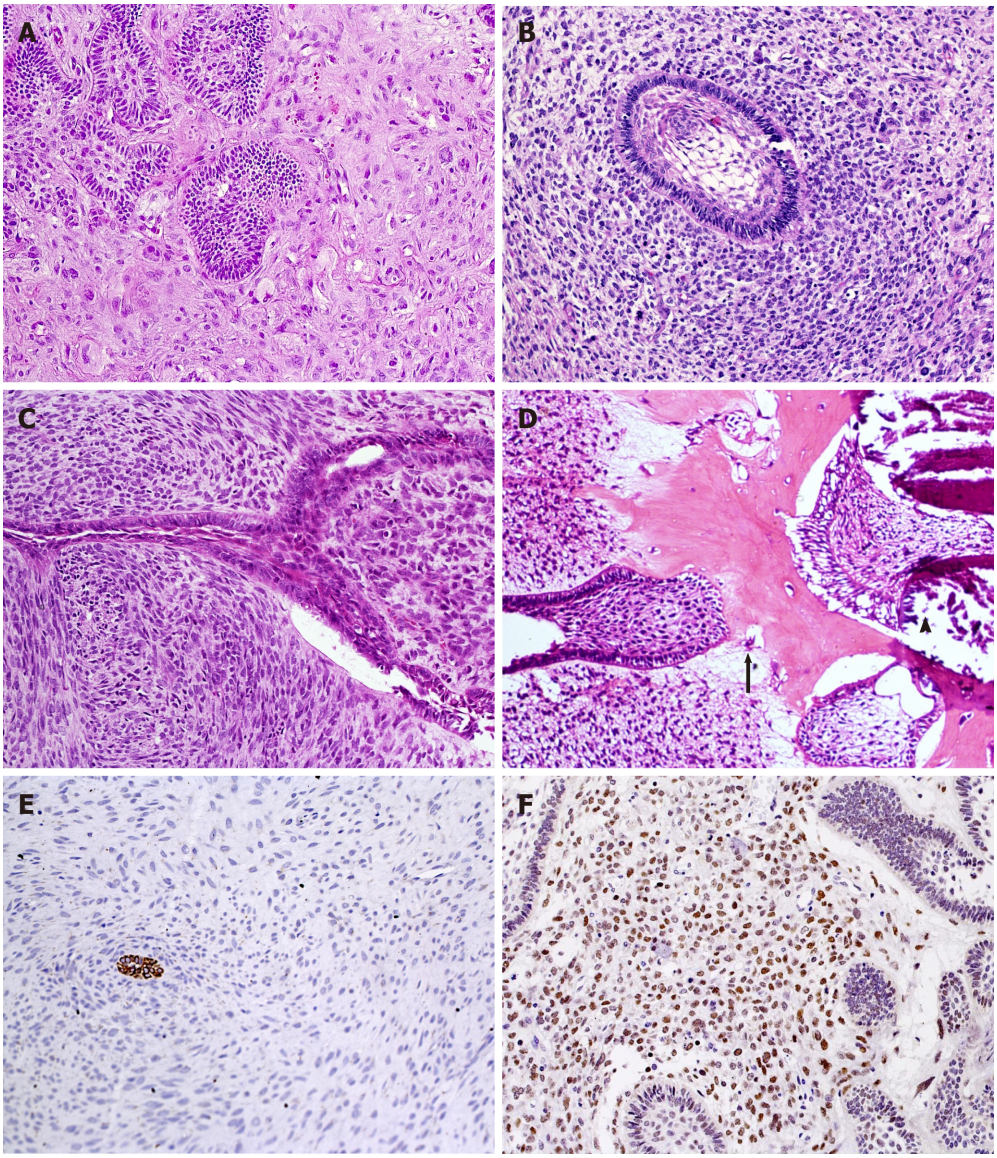


Figure 3 Histopathological aspects of ameloblastic fibromas and ameloblastic fibro-odontosarcoma. Marked pleomorphism and atypia in mesenchymal cells (HE, 20×) (A-C). A: Several mitotic figures, nuclear hyperchromatism and multinucleated and aberrant cells are seen in a highly pleomorphic sarcomatous component of ameloblastic fibromas, while epithelial islands remain benign; B: A follicular benign epithelial island is surrounded by hypercellularized sarcomatous proliferation; C: Malignant mesenchymal tissue resembling a storiform pattern and haphazard disposition of sarcomatous cells around an epithelial branching cord; D: Production of enamel matrix (arrowhead) and dentinoid (arrow) as well as malignant mesenchymal tissue (left side) are components of this ameloblastic fibro-odontosarcoma (A-D: HE, 20×); E: Cytokeratins can help to localize odontogenic epithelial cells within dominant sarcomatous proliferation (IHC for AE1/AE3, 20×); F: Most mesenchymal malignant cells show nuclear positivity for p53 antigen (IHC for p53, 20×).

[38], the NRAS p.Gln61Lys mutation was also detected in one AFS case, and another case was wild type.

DISCUSSION

AF, AFD and AFO present similar clinical, radiographic and microscopic features and were accepted as different entities until the 2017 WHO classification, which considers these entities as representing diverse stages of maturation of a developing odontoma, as suggested by Cahn and Blum in the past[39]. In summary, we suggest that the lesions referred to as AFD and AFO are more likely "developing odontomas" and are no longer considered mixed odontogenic tumors, as in the previous classifications of odontogenic tumors.

We consider that the WHO classification of these tumors in 2017 is unclear because it states that it is not possible to differentiate histologically between AF (true neoplasms) and early-stage odontomas before they differentiate and mature; therefore,

the existence of both lesions is accepted. However, no evidence has shown that AF matures to odontoma. If maturation of AF occurred, it would be expected that AF (immature lesion) would be diagnosed at an earlier age than AFO (mature lesion). However, an opposite trend has occurred: as the mean age of diagnosis of AFO is 9.6 years and that of AF is 14.9 years. However, because several AFOs have been reported in children in areas of odontogenesis, some cases might represent odontomas. Nevertheless, some cases of AFD/AFO arise in age groups that are not consistent with a hamartoma: from the 45 reviewed cases of AFD, 17 cases (37.7%) were aged ≥ 20 years, and 7 cases (15.5%) were aged ≥ 30 years, 4 of which were in the sixth and seventh decades of life.

The terms AFD and AFO were practically discarded from the latest WHO tumor classification, considering that once hard tissues are produced, these tumors are more likely to form odontomas[2,40]. Nevertheless, in this WHO classification, AFD and AFO might conceptually be neoplastic when reaching an exceptionally large size but without establishing a measure for this statement[2].

To avoid concepts that may be confusing and that are not appropriately supported by scientific evidence, we suggest not using the term developing odontomas and simply continuing to use odontomas if the clinical, radiographical and microscopic characteristics support this well-established diagnosis. Cases of a typical odontoma associated with AF could be termed AF associated with odontoma because odontomas can eventually be associated with other odontogenic tumors.

We accept that some cases are difficult to classify as AFO or odontoma because of the large amounts of hard dental tissues and because some cases of odontoma have been diagnosed as AFO. However, a cut off could be considered based on the proportion of hard and epithelial-ectomesenchymal tissues, as well as clinical (size, location, age, and clinical behavior) and radiographic features.

No evidence exists that all AF/AFD/AFO are "developing odontomas" because each of these tumors has its own clinicopathological features. AF is a well-recognized entity, and it should also be emphasized that no evidence is available that AF matures and forms small or large amounts of hard dental tissues, even in cases of recurrence.

AFD has no potential to produce enamel/enameloid; therefore, it cannot mature to an odontoma. However, some AFOs can produce large amounts of hard dental tissues and may mimic radiographically and microscopically odontomas; nevertheless, most AFOs present relatively few calcified areas. We reviewed 82 cases in the English literature with adequate radiographic documentation, most of which had small amounts of hard tissues: 59.8% presented few opacities or radiolucent images, 13.4% showed a higher number of scattered opacities, and only 26.8% presented a single opaque mass similar to odontoma. Even considering these cases rich and poor in calcified dental tissues diagnosed as AFO, evidence exists that cases poor in dental calcified structures evolve to those that mimic odontomas.

Recent molecular studies have shown genetic differences (principally, *BRAF*V600E mutation) between odontoma (BRAF wild type) and a subset of AF, AFD, AFO and most AFS, supporting that these lesions may represent distinct entities with a neoplastic nature[36-38].

In summary, we propose to continue to use the classical terms AFD and AFO because it is part of the 2017 WHO classification for malignant counterparts. This recommendation can be relevant for future clinical, microscopic and molecular studies to better clarify the subject and better understand the biology of these interesting odontogenic tumors.

Several aspects support the neoplastic nature of AF, AFD and AFO, such as their biological behavior, significant frequency of *BRAF* mutation, age of occurrence, amount of hard tissue and potential for malignant transformation into odontogenic sarcomas with or without the production of dental hard tissues. Among the 18 cases of AFDS/AFOS reported in the literature, 6 were related to a preexisting AFO, and this malignant transformation would not be expected in a hamartomatous lesion as a developing odontoma. The 2017 WHO classification accepts AFS, AFDS and AFOS as entities, and they can be de novo or derived from AF. This inconsistency in the nomenclature between benign and malignant corresponding tumors probably occurred because the topics of "odontogenic sarcomas" and "ameloblastic fibroma" were written by different authors in the 2017 WHO classification.

Odontogenic carcinosarcoma was added to the 2017 WHO classification based on 6 case reports, considering that it may arise de novo or can be derived from previous AF or AFS. However, we also found in the literature that, in two cases, ameloblastoma and malignant ameloblastoma were reported as the preceding tumors (Table 3). In contrast to AFS, in which metastasis is rare, 33% (3 cases) of odontogenic/ameloblastic carcinosarcomas presented biphasic metastasis (epithelial and sarcomatous

Table 3 Main data of 9 cases reported of ameloblastic/odontogenic carcinosarcoma in the literature

Case	Ref.	Sex/age	Location	Preexisting tumor	Progression
1	Tanaka *et al*[91]	M/63	Maxilla	Malignant ameloblastoma	Recurrence, metastasis and death
2	Slama *et al*[92][1]	F/26	Mandible	AF	Metastasis and death
3	Kunkel *et al*[3]	M/52	Mandible	No	Recurrence, metastasis and death
4	DeLair *et al*[93]	F/19	Mandible	AF	No recurrence
5	Chikosi *et al*[94]	F/9	Mandible	Ameloblastoma	Recurrence and death
6	Kim *et al*[4]	M/61	Mandible	No	No recurrence
7	Dos Santos *et al*[95]	M/42	Maxilla	No	Unknown
8	Soares *et al*[96]	M/22	Mandible	No	No recurrence
9	Soares *et al*[96]	F/19	Mandible	Rhabdomyosarcoma (parotid region)[2]	Post-surgical systemic infection and death

[1]Article in French, abstract in English.
[2]Thirteen years before, treated with surgical resection followed by radiotherapy.
M: Male; F: Female; AF: Ameloblastic fibroma.

components), and 5 of 9 cases resulted in death[3]. Thus, this entity was recently recognized at the present WHO classification. Immunohistochemically, positivity for p53 and a Ki-67 index > 45% in both carcinomatous and sarcomatous components can be useful to confirm the diagnosis[2].

It is reasonable to consider that basic benign and malignant neoplasms are AF and AFS and that the presence of small amounts of dental hard tissues does not significantly alter the biological characteristics and clinical behaviors of these entities [1,13]. Although not clearly established, the presence and higher amount of hard tissues may indicate less aggressiveness and possibly lower potential of malignant transformation. In this context, AFO should have a better prognosis than AF/AFD, with a lesser tendency for malignant transformation. AFDS/AFOS seem to have a similar rate of recurrence as AF; however, the metastasis and mortality indexes seem to be higher in AFSs. Additionally, the number of cases of AFD/AFO and AFDS/AFOS reported is very small, making comparisons of these tumors with AF/AFS difficult.

Reports of AFSs have been present for several years, possibly as AF/AFO/AFD that have suddenly followed an aggressive course before being treated, indicating a possible malignant transformation[25,41].

CONCLUSION

In summary, we reviewed the principal clinical, histopathological and molecular characteristics of AF, AFD and AFO and their malignant counterparts. Odontogenic/ameloblastic carcinosarcoma was cited because, according to reports, it can arise from preexisting AF. We consider that the recent 2017 WHO classification does not clarify the subject when considering AFD and AFO as developing odontomas. According to the clinical, radiographical, histopathological and molecular features of the cases reviewed, we suggest that AFD and AFO should continue to be considered benign neoplasms. Thus, the nomenclature of these mixed benign odontogenic tumors would be congruent with the classification of ameloblastic/odontogenic sarcomas. Additionally, further studies are warranted to compare these interesting odontogenic tumors and finally better clarify and understand their similarities and differences.

ARTICLE HIGHLIGHTS

Research background

Ameloblastic fibromas and ameloblastic fibrosarcomas are rare odontogenic tumors, and controversy exists in the classification of cases presenting hard-tissue production: Ameloblastic fibrodentinoma (AFD) and ameloblastic fibro-odontoma (AFO). These

cases are currently considered "developing odontomas" (hamartomatous lesions). There is still controversy as to whether they are true hamartomas or neoplasms.

Research motivation

The authors consider that the recent 2017 WHO classification does not clarify the subject when considering AFD and AFO as "developing odontomas". According to the clinical, radiographical, histopathological and molecular features of the cases reviewed, we suggest that AFD and AFO should continue to be considered benign neoplasms.

Research objectives

The objective was to analyze the clinicopathologic features of these lesions and discuss the changes in the 2017 WHO classification.

Research methods

For this systematic review an electronic literature search was performed in the PubMed/MEDLINE database. An exhaustive search was made of all the existing information on these mixed odontogenic tumors.

Research results

Several aspects of AFO and AFD, such as biological behavior, age of occurrence, amount of hard tissue, and potential for malignant transformation into odontogenic sarcomas, support the neoplastic nature in most of the reported cases.

Research conclusions

Considering the clinical, radiographic, histopathological, and molecular characteristics of odontogenic lesions with hard tissue production, we suggest that these types of lesions should continue to be recognized as odontogenic tumors by maintaining the classically used terms. This recommendation will be relevant for future clinical, microscopic, and molecular studies to better understand the biology of these interesting odontogenic tumors. This new information will be relevant for the clinical conduct to be followed in these tumors.

Research perspectives

Future research should be focused on the comparative molecular study between these odontogenic neoplasms and odontomas; trying to clarify molecular differences between neoplasia and hamartoma.

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SYSTEMATIC REVIEWS

Carcinosarcoma of gallbladder: A world review

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Abstract

BACKGROUND

Gallbladder carcinosarcoma is a rare hepatobiliary tumor comprising of both carcinomatous and sarcomatous components. Due to its rarity, the literature with regards to the topic is scarce and currently lacking, spanning less than 100 cases.

AIM

To summarize the current literature on gallbladder carcinosarcoma.

METHODS

A literature review was performed on the PubMed database using the keywords "Gallbladder" AND "Carcinosarcoma" from 1970 to 2021. Additionally, similar searches were performed on MEDLINE and Web of Science.

RESULTS

Risk factors noted include female gender, gallstones and chronic cholecystitis. In the absence of any diagnostic biochemical testing or tumor markers, imaging modality serves as the key initial impression tool, which can be histologically confirmed only post-resection. While surgery is the only curative option, the use of adjunctive chemotherapy has been considered on top of excision in recent years, with some success.

CONCLUSION

While this study has taken steps to bridge the gap in the literature, more cases should be reported to further ascertain the current associations and management potential for gallbladder carcinosarcoma.

Key Words: Carcinosarcoma; Gallbladder; Gallstone; Malignancy; Carcinoma; Sarcoma

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Core Tip: Gallbladder carcinosarcoma (GBCS) while rare, is an important histological subtype of gallbladder malignancy as it is associated with poor prognosis. Most GBCS patients tend to present late. As of now, the primary method of diagnosis is that of a pathological analysis with the main stay of treatment being surgical excision. Furthermore, the clinical diagnosis of GBCS remains extremely challenging given its seemingly nonspecific clinical features. We aim to provide an in-depth world review of the known cases of GBCS in order to identify unifying features of the disease and to assess effective management strategies that have been employed by clinicians.

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INTRODUCTION

Gallbladder carcinosarcoma (GBCS) is defined by the presence of both carcinomatous and sarcomatous components in the tumor, making it a rarity even amongst the uncommon gallbladder cancer family[1]. While its history is deep-rooted, with the first case being reported by Karl[2] in 1907, less than 100 cases have been reported since. In 2008, Zhang *et al*[3] sought to collectively analyze the 70 cases in the literature at that time. However, Zhang *et al*[3] noted the need for a larger scale case series to provide more information on the neoplasm for better accuracy and reliability. Since then, there has been a gap in the literature for such an analysis (Figure 1). This study aims to fill this gap by providing a comprehensive overview of GBCS.

MATERIALS AND METHODS

A literature review was performed on the PubMed database using the keywords "Gallbladder" AND "Carcinosarcoma" from 1970 to 2021. Additionally, similar searches were performed on Medline and Web of Science. The last search was performed on January 31, 2021. After removing duplicate results from similar databases, the search yielded 105 articles: 16 non-English and non-Japanese studies and 12 unrelated topics (animal studies, gallbladder carcinoma and non-gallbladder pathology) were excluded. Out of the remaining 77 articles, seven were not case reports or case series on GBCS and thus excluded. The remaining 70 articles including 76 patients were included in the final analysis (Table 1)[1,4-72]. Article filtering and exclusion was done according to PRISMA guidelines (Figure 2). Data extracted included study year, age and gender of the patient, clinical presentation, risk factors, laboratory investigations, tumor markers, the ultrasound imaging findings, location of the lesion within the gallbladder, size of the lesion, initial diagnosis, method of confirming the diagnosis, immunohistochemical results (vimentin, cytokeratin, Ki-67), management and prognosis of the patient. Kaplan-Meier survival curves were compared between lesions larger than 5 cm and those smaller than 5 cm as data by Zhang *et al*[3] suggested that tumors smaller than 5 cm had better survival. For all statistical tests, a P value of 0.05 was used to determine statistical significance.

RESULTS

Seventy-eight patients with a mean age of 66.4 years (range: 40-91 years) were reported during the study period. The patients were predominantly female (n = 55, 72.4%) with a gender ratio of 2.62. Nine patients (11.8%) had chronic cholecystitis, and 1 patient each had hepatitis C and abnormal pancreaticobiliary maljunction (APBJ). Of those who reported the presence of gallstones, a majority noted the presence of gallstones (n = 35/42, 83.3%). The majority of patients complained of abdominal pain (n = 58, 76.3%), most of which was localized to the right upper quadrant. Twenty-two patients (28.9%) presented with constitutional symptoms (either unexplained loss of

Table 1 Summary of 76 reported cases of gallbladder carcinosarcoma from 1970 to 2021

No.	Year	Ref.	Age/sex	Risk factors for GB CA (stones)	Clinical presentation	Liver function tests	Position of CA	Tumor markers (CEA, AFP, CA 19-9)	Size (mm)	Initial diagnosis	Confirmatory diagnosis (mode)	Stage (UICC)	Survival (mo)	IHC positives	Further management
1	2020	Khurram *et al* [4]	64/F	No stones	RUQ pain, intermittent fever, abdominal distension	AST, GGT elevated	Fundus	Normal	132 × 97 × 110	Hepatic abscess	Cholecystectomy	NA	NIL mentioned	CK	NA
2	2020	Ayoub *et al*[5]	66/M	NA	RUQ pain	Normal	Body	Normal	150 × 80 × 60	Gallbladder malignancy	Cholecystectomy and lymphadenectomy	IVA	12+	Vimentin, CK	NA
3	2020	Kaneko *et al*[6]	70/F	NA	Obstructive jaundice	NA	NA	Normal	110 × 70 × 34	Gallbladder malignancy	Cholecystectomy	NA	44+	CK, Ki-67	NA
4	2020	Siddiqui *et al*[7]	57/M	NA	Abdominal pain, nausea, LOW, LOA	ALP, total bilirubin elevated	Fundus	NA	620	Gallbladder malignancy	ERCP (unsuccessful), PTC with internal-external biliary drainage catheter	NA	NA	Vimentin	NA
5	2020	Mochizuki *et al* [8]	88/F	Gallstones	Chills, tremors, vomiting	NA	Body	NA	60 × 25	Acute cholecystitis	Cholecystectomy	NA	10 +	Ki-67	NA
6	2019	Varshney *et al* [9]	50/M	Gallstones	RUQ pain, obstructive jaundice	AST, ALT, bilirubin elevated	Fundus	Normal	65 × 55	Gallbladder malignancy	radical cholecystectomy with standard lymphadenectomy	NA	6+	Vimentin, CK	Adjuvant chemotherapy
7	2019	Aldossary *et al* [10]	40/M	Gallstones	RUQ pain	Normal	Entire gallbladder	Normal	115 × 92 × 50	Gallbladder malignancy	Open lap, radical cholecystectomy, extended R hemi w IC anastomosis, liver resection	IVB	6	Vimentin	Adjuvant chemotherapy
8	2019	Aldossary *et al* [10]	52/F	No stones	RUQ pain	ALT, AST elevated	Fundus	CA19-9 level of 154.3 IU/mL, with normal levels of AFP and CEA	136 × 120 × 95	Gallbladder Malignancy	Open lap, radical CCY, transverse chole, Roux en Y + distal gastrectomy	IVB	3	Vimentin, CK	NA
9	2019	Aldossary *et al* [10]	62/F	Gallstones	RUQ pain, nausea, anorexia	Normal	Body	Normal	27 × 9	Gallbladder malignancy	Lap CCY	II	86+	Vimentin, CK	Adjuvant chemotherapy

10	2019	Alratroot *et al* [11]	52/F	Xanthogranulomatous cholecystitis	RUQ pain	GGT elevated	Fundus	CA 19-9 154.33 IU/mL	110 × 60	Gallbladder malignancy	Laparotomy with radical cholecystectomy, transverse colectomy, distal gastrectomy, omentectomy and liver bed resection	III	1.5+	Vimentin, CK	Adjuvant chemotherapy
11	2019	Matsubayashi *et al*[12]	72/F	Pancreaticobiliary maljunction	RUQ pain	ALP, GGT elevated	Entire gallbladder	Normal	90 × 85	Gallbladder malignancy	Laparotomy and extended cholecystectomy	IIIA	73+	Vimentin, CK	NA
12	2018	Doniparthi *et al* [13]	49/M	NA	Epigastric pain	AST, ALT, lipase elevated	NA	Normal	32	Acute cholecystitis	Lap cholecystectomy, followed up by robotic liver resection and lymphadenectomy	NA	NA	NA	NA
13	2018	Koustav *et al* [14]	40/F	NA	RUQ pain		NA	CA19-9 elevated	43 × 51	Gallbladder malignancy	Staging laparoscopy + extended cholecystectomy	NA	NA	NA	NA
14	2018	Trautman *et al* [15]	73/F	Chronic cholecystitis	Abdominal distension, constipation, vomiting, LOW	AST, ALT, ALP elevated	NA	Beta-HCG elevated		Gallbladder malignancy	Diagnostic laparoscopy	NA	0.5	Vimentin	Palliative (NM)
15	2017	Furuya *et al*[16]	61/F	NA	RUQ pain	Normal	NA	Normal	15 × 15	Chronic cholecystitis with stone	Cholecystectomy	NA	NA	NA	NA
16	2016	Hu *et al*[17]	68/F	Cholelithiasis	RUQ pain, fever	Normal	Body	CA19-9 elevated	16 × 15 × 13	Gallbladder malignancy	Cholecystectomy	NA	1	NA	NA
17	2016	Cruz *et al*[18]	52/F	Gallstones	RUQ pain	ALT AST elevated	Entire gallbladder	Normal	170 × 125	Gallbladder malignancy	Cholecystectomy	NA	1	Vimentin, CK	Palliative (NM)
18	2016	Dong *et al*[19]	61/M	NA	Abdominal distension	NA	NA	Normal	180	Gallbladder malignancy	Resection (not specified)	NA	NIL mentioned	Ki-67	NA
19	2016	Gupta *et al*[20]	46/F	NA	RUQ pain	NA	Fundus	All normal	350 × 250 × 200	Gallbladder malignancy	Radical cholecystectomy with hepato-duodenal ligament lymph node clearance and segment 4b/5 liver resection	NA	15 (still alive)	Vimentin, CK	Adjuvant chemotherapy
20	2016	Wong *et al*[21]	52/F	NA	Abdominal pain	NA	Entire gallbladder	CA19-9 elevated	75	NA	Autopsy	III	6	Vimentin, CK	Adjuvant chemotherapy
21	2016	Ansari *et al*[22]	50/F	NA	RUQ pain	Normal	Entire gallbladder	Normal	50 × 40	NA	Radical cholecystectomy	II	13 mo (still alive)	Vimentin, CK, Ki-67	Adjuvant chemotherapy
22	2015	Gao *et al*[23]	62/M	Chronic cholecystitis	RUQ pain	Normal	Entire gallbladder	Normal	50 × 40	Gallbladder malignancy	Simple cholecystectomy	II	0	Vimentin, CK	NA
23	2015	Tonouchi *et al* [24]	87/M	No stones	Abdominal pain	NA	NA	NA	60 × 55	Diffuse peritonitis	Cholecystectomy with partial transverse colectomy around the fistula	NA	Lost to follow-up	Vimentin, CK	NA

24	2015	Faujdar *et al* [25]	60/F	NA	RUQ pain, fever	Normal	Entire gallbladder		120 ×70 × 60	Gallbladder malignancy	Cholecystectomy	NA	60+	Vimentin, CK	NA
25	2014	Wada *et al*[26]	68/M	NA	Right flank pain	GGT elevated	NA	Normal	85 × 70	Gallbladder malignancy	Extended right hepatectomy with portal thrombectomy with hepatoduodenal ligament lymphadenectomy	NA	51+	Vimentin, CK, Ki-67	Adjuvant chemotherapy
26	2014	Kishino *et al* [27]	70s/F	NA	Referred for suspected GB cancer (presenting complaint not mentioned)	NA	Fundus	NA	68	Gallbladder malignancy	Cholecystectomy	NA	1.5+	Vimentin, CK	NA
27	2013	Wang *et al*[28]	68/F	Chronic cholecystitis, cholecystolithiasis	RUQ pain, jaundice, fever	ALT, ALP elevated	NA	CEA, CA19-9, AFP elevated	100 × 70 × 50	Gallbladder malignancy	Cholecystectomy with liver segmentectomy (S4a+S5) and a lymph node dissection, followed by resection of the extrahepatic bile duct and a Roux-en-Y type hepatic cholangiojejunostomy	NA	6+	Vimentin, CK	NA
28	2013	Khanna[29]	45/F	NA	RUQ pain	Normal	Body		60 × 40	Gallbladder malignancy	Laparotomy and simple cholecystectomy with wedge resection	NA	3	Vimentin, CK, Ki-67	NA
29	2013	Li *et al*[30]	64/M	Chronic cholecystitis	RUQ pain	NA	NA	CEA, CA19-9 elevated	40 × 30 × 30	NA	Cholecystectomy, R hemicolectomy, resection of multiple hepatic metastases	NA	3+	Vimentin, CK, Ki-67	NA
30	2012	Kim *et al*[31]	72/F	Gallstones	Abdominal pain	Normal	Fundus	Normal	65 × 45 × 45	Gallbladder malignancy	Radical cholecystectomy with wedge resection of liver combined with hepatoduodenal ligament lymphadenectomy	NA	4	NA	Adjuvant chemotherapy
31	2012	Kim *et al*[31]	81/M	NA	Epigastric pain	Normal	Fundus	Normal		Gallbladder malignancy	Cholecystectomy with liver segmentectomy (S4a,5) and lymph node dissection	NA	13	Vimentin, CK	NA
32	2012	Sadamori *et al* [32]	80/M	NA	RUQ pain, fever		Entire gallbladder		76 × 27	Gallbladder malignancy	Cholecystectomy with liver segmentectomy (S4a and S5) and lymph node dissection	NA	2+		Adjuvant chemotherapy
33	2012	Kataria *et al*[33]	55/F	NA	RUQ pain	Normal	Fundus	Normal	70 × 50 × 30	NA	Cholecystectomy, wedge resection of liver with resection of transverse colon and paraduodenal lymph node	NA	6	Vimentin, CK	NA
34	2012	Parreira *et al* [34]	59/F	NA	RUQ pain	Normal	NA	NA	NA	NA	Conventional cholecystectomy	NA	2	NA	NA

35	2012	Park *et al*[35]	77/F	NA	RUQ pain	AST, ALT elevated	NA	CA19-9, CA-125 elevated	78 × 55 × 12	Gallbladder malignancy	Laparotomy, followed by cholecystectomy and lymph node dissection	IIIB	10+	NA	NA
36	2012	Ishida *et al*[36]	62/F	NA	Incidental finding on radiograph for left calcaneal fracture	Normal	Entire gallbladder	Normal	52 × 38	Gallbladder malignancy	Open cholecystectomy	NA	8	NA	NA
37	2011	Lee *et al*[37]	77/F	No stones	RUQ pain	Not mentioned	Body	CA19-9, CA-125 elevated	80 × 70 × 30	Gallbladder malignancy	Cholecystectomy	NA	1.5+	Vimentin, CK	NA
38	2011	Pu *et al*[38]	59/F	Cholecystolithiasis	RUQ pain, fever	Normal	Body	CA19-9 elevated	120 × 25 × 60	Gallbladder malignancy	Exploratory laparotomy, followed by radical LN resection and hepatocholangojejunostomy Roux-En-Y	II	0	CK	Adjuvant chemotherapy
39	2011	Krishnamurthy *et al*[39]	83/M	No stones	Abdominal pain	NA	NA	NA	NA	NA	Laparoscopic cholecystectomy	NA	48+	Vimentin, CK	NA
40	2009	Kohtani *et al* [40]	84/M	Chronic cholecystitis	RUQ pain	Serum glutamic oxaloacetic transaminase, GGT elevated	Neck	NA		Gallbladder malignancy	Open cholecystectomy	II	3+	Vimentin, CK, Ki-67	Adjuvant chemotherapy
41	2009	Agarwal *et al* [41]	60/F	NA	RUQ pain, fever	Normal	Neck	NA	70 × 50 × 40	Gallbladder malignancy	Staging laparoscopy, laparotomy, simplex cholecystectomy	NA	24+	Vimentin, CK	NA
42	2009	Magata *et al*[42]	78/F	NA	RUQ pain	NA	Body	CEA elevated	115 x 40 x 35	Gallbladder malignancy	Whole-layer cholecystectomy with regional lymph node dissection	NA	6+	Vimentin, CK	NA
43	2009	Shimada *et al* [43]	69/M	Choledocholithiasis	Fever	Normal	Entire gallbladder	AFP elevated	90 × 50	Gallbladder malignancy	Laparotomy, cholecystectomy, lymph node dissection	NA	54+	Vimentin, CK, Ki-67	NA
44	2009	Uzun *et al*[44]	70/M	NA	RUQ pain	Normal	Fundus	Normal	100 × 60 × 30	Gallbladder malignancy	Radical cholecystectomy, wedge resection of liver-gallbladder bed with hepatoduodenal ligament lymphadenectomy	NA	8	CK, Ki-67	NA
45	2006	Kubota *et al*[45]	72/M	NA	RUQ pain, fever	AST, ALT, ALP elevated	NA	Normal	70 × 55 × 40	Gallbladder malignancy	En bloc resection of the gallbladder and segments 4a and 5 of the liver, partial colectomy, and lymph node dissection	NA	6	NA	NA
46	2005	Akatsu *et al*[46]	76/F	Gallstones	Incidental	Normal	NA	Normal		Gallbladder	Extended cholecystectomy,	NA	2	Vimentin,	NA

					finding on follow-up for cholelithiasis					malignancy	liver 4b and 5 resection			CK	
47	2005	Huguet *et al* [47]	64/F	Cholecystitis	RUQ pain, fever	Normal	Entire gallbladder	Normal	120 × 100 × 70	Gallbladder malignancy	A cholecystectomy with wedge resection of the gallbladder fossa (involving liver segments 4 and 5), extrahepatic bile duct excision, non–pylorus-preserving pancreaticoduodenectomy with excision of 15 cm of proximal jejunum, and right hemicolectomy	NA	60	Vimentin, CK	NA
48	2005	Sodergren *et al* [48]	64/F	NA	Malaise and LOA	ALP Elevated	NA	NA	20 × 12 × 12	NA	Extrahepatic radical bile duct resection with hepatic and coeliac lymph node clearance followed by right hepaticodochojejunostomy to a jejunal Roux loop	NA	5	Vimentin, CK	NA
49	2005	Sodergren *et al* [48]	60/F	NA	Painless jaundice	NA	NA	NA	90	Gallbladder malignancy	Cholecystectomy and extrahepatic bile duct resection with lymph node clearance	NA	2	Vimentin, CK	Palliative (NM)
50	2004	Takahashi *et al* [49]	84/F	NA	RUQ pain	NA	Body	CEA, CA19-9 elevated	84 × 40 × 30	Gallbladder malignancy	Cholecystectomy and transverse colon partial colectomy	NA	2	Vimentin, CK	
51	2003	Kim *et al*[50]	61/F	No stones	RUQ pain	Normal	Neck	Normal	45 × 40 × 40	Gallbladder malignancy	Cholecystectomy with common bile duct resection	NA	2	Vimentin	Palliative (NM)
52	2002	Al-Sheneber *et al*[1]	68/F	Acute cholecystitis, gallstones	RUQ pain	Normal	NA	CEA elevated	148 × 80	Gallbladder malignancy	CT guided needle biopsy of the upper abdominal mass	NA	7	Vimentin, CK	NA
53	2002	Hotta *et al*[51]	53/M	Chronic cholecystitis, gallstones	RUQ Pain	Normal	NA	Normal	1100	Gallbladder malignancy	Cholecystectomy with resection of subsegmentectomy of liver S5 and a resection of transverse colon at the second operation	II	2	NA	Adjuvant chemotherapy
54	2002	Ajiki *et al*[52]	69/F	Gallstones, left renal tumor	Epigastric pain	Normal	NA	CA19-9 elevated	NA	Double cancers of the left kidney and gallbladder	Left renal excision, cholecystectomy with liver segmentectomy (S4a, S5), and lymph node dissection	NA	NA	NA	Adjuvant chemotherapy
55	2000	Yavuz *et al*[53]	50/F	NA	RUQ pain	NA	Body	NA	80 × 60 × 60		Exploratory laparotomy -> cholecystectomy, liver wedge biopsy	NA	NA	NA	NA

56	1999	Eriguchi *et al* [54]	65/F	Gallstones	RUQ pain	Normal	Entire gallbladder	NA		Gallbladder malignancy	Cholecystectomy	I	16+	NA	NA
57	1997	Ryś *et al*[55]	67/F	Gallstones	Abdominal pain, LOW	NA	Fundus	NA	10 × 15	Gallbladder malignancy	Hemicolectomy and cholecystectomy	NA	2	Vimentin	NA
58	1996	Nakagawa *et al* [56]	60/F	NA	Abdominal pain, fever	Normal	Body	NA	70 × 40	Gallbladder malignancy	Mass reduction surgery	NA	NA	NA	NA
59	1994	Fagot *et al*[57]	83/F	Gallstones	Vomiting, fever, right RHC pain	Total bilirubin elevated	Fundus	NA	45	NA	Surgery (not defined)	NA	12+	NA	NA
60	1992	Nakazawa *et al* [58]	63/F	NA	Nausea	Normal	Body	Normal	30 × 30	Gallbladder malignancy	Pancreaticoduodenectomy	NA	NA	NA	NA
61	1990	Ishihara *et al* [59]	58/F	NA	Abdominal pain	NA	Fundus	NA	50 × 80	Gallbladder malignancy	Cholecystectomy	NA	11+	Vimentin	NA
62	1988	Lumsden *et al* [60]	81/F	Gallstones	RUQ pain, LOW, LOA	Total bilirubin, ALP, GOT elevated	NA	NA	50 × 20 × 20	Biliary neoplasm	Cholecystectomy	NA	12+	NA	NA
63	1987	Hasegawa *et al* [61]	61/M	NA	RUQ pain	Normal	Entire gallbladder	NA	150	Gallbladder malignancy	Resection (not specified)	NA	6	NA	NA
64	1987	Herrera-Goepfert *et al* [62]	60/F	Gallstones	Abdominal pain, jaundice, LOW		Entire gallbladder	NA	70 × 40	Gallbladder malignancy	Autopsy	NA	NA	NA	NA
65	1986	Inoshita *et al* [63]	53/M	Gallstones	RUQ pain, jaundice	Total bilirubin, ALP, GOT, GPT elevated	Neck	NA	NA	Choledocholithiasis	Open laparotomy	NA	17	NA	NA
66	1985	Lopez *et al*[64]	78/F	No stones	Anorexia, LOW	Normal	NA	NA	NA	Gallbladder empyema	Open laparotomy	NA	NA	NA	NA
67	1984	Born *et al*[65]	90/F	Gallstones	Anorexia, nausea, vomiting	Amylase elevated	NA	NA	150 × 150 × 10	NA	Exploratory laparotomy	NA	3	NA	NA
68	1982	von Kuster *et al* [66]	91/F	Gallstones	RUQ pain, fever	GOT, ALP elevated	NA	NA	20	Gallbladder empyema	Exploratory laparotomy	NA	0	NA	NA
69			77/F	NA	Bleeding in GI tract (lower)	Normal	NA	NA	30	NA	Exploratory laparotomy	III	31+	NA	NA
70	1982	Aldovini *et al* [67]	75/F	Gallstones	Abdominal pain	ALP, SGT elevated	NA	NA	90	NA	Cholecystectomy	NA	8+	NA	NA
71	1982	Yamagiwa *et al* [68]	78/F	NA	RUQ pain	NA	NA	NA	NA	NA	NA	NA		NA	NA

72	1980	Mansori *et al* [69]	81/M	Gallstones	Abdominal pain	GOT, ALP elevated	NA	NA	NA	NA	Exploratory laparotomy	NA	0.5	NA	NA
73	1973	Higgs *et al*[70]	77/M	Gallstones	Jaundice	ALP, GOT elevated	NA	NA	NA	NA	Cholecystectomy and CBDE	NA	1	NA	NA
74	1971	Mehrotra *et al* [71]	45/F	Gallstones	RUQ pain	Normal	Neck	NA	50 × 40 × 30	NA	Open laparotomy	NA	4	NA	NA
75	1970	Appelman *et al* [72]	91/M	Gallstones, chronic cholecystitis	Obstructive jaundice	AST, ALT, ALP elevated	Fundus	NA	NA	Pancreatic cancer	Autopsy	NA	0.5	NA	NA
76	1970	Appelman *et al* [72]	75/F	Gallstones, chronic cholecystitis	RUQ pain	ALP elevated	Fundus	NA	50 × 50 × 20	NA	Cholecystectomy	NA	1	NA	NA

GBCA: Gallbladder cancer; CEA: Carcinoembryonic antigen; AFP: Alpha-fetoprotein; CA 19-9: Carbohydrate antigen 19-9; UICC: Union for International Cancer Control; IHC: Immunohistochemistry; RUQ: Right upper quadrant; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; ERCP: Endoscopic retrograde cholangiopancreatography; PTC: Percutaneous transhepatic cholangiogram; CCY: Cholecystectomy; NA: Not available; NM: Not mentioned; NIL: None; CR: Complete response; LOA: Loss of appetite; LOW: Loss of weight; R hemi w C: Right hemihepatectomy with cholecystectomy; M: Male; F: Female; Lap: Laparoscopic; Chole: Cholecystectomy; LN: Lymph node; CT: Computerized tomography; RH: Right hepatectomy; GI: Gastrointestinal; CBDE: Common bile duct exploration; HCG: Human chorionic gonadotropin; SGOT: Serum glutamic-oxaloacetic transaminase; SGPT: Serum glutamic-pyruvic transaminase.

weight, anorexia or lethargy). Nineteen patients (25.0%) had nausea and vomiting, and 13 patients (17.1%) were febrile. Two patients (2.6%) were asymptomatic when diagnosed.

Liver function test was the common serum biochemical test reported (n = 57). Deranged liver function tests were reported in 25 (43.9%) patients. Tumor markers were variably reported. The following tumor markers were elevated: carbohydrate antigen 19-9 (CA19-9) (n = 9/27, 33.3%), carcinoembryonic antigen (n = 5/27, 18.5%) and alpha-fetoprotein (n = 2/12, 16.6%) in some patients. Also, CA-125 was elevated in 2 patients.

Forty-three patients had the location of the gallbladder tumor reported. Fundus was the most common location (n = 15, 34.9%), followed by body (n = 10, 23.3%) and neck (n = 5, 11.6%). In 14 patients (32.5%), the tumor filled the entire gallbladder lumen, and thus exact position could not be determined. Fifty-nine patients had initial diagnosis reported. Out of these 59 patients, gallbladder malignancy was the primary diagnosis in the majority of patients (n = 49, 83.1%). Ten patients (16.9%) were initially diagnosed with other pathologies: cholelithiasis (n = 1), cholecystitis (n = 3), gallbladder empyema (n = 2), diffuse peritonitis (n = 1), pancreatic cancer (n = 1), biliary neoplasm (n = 1) and pyogenic liver abscess (n = 1).

Confirmation of diagnosis was reported in all but 1 patient (n = 75). It was mostly done *via* surgical resection, either diagnostic cholecystectomy or laparotomy (n = 70, 93.3%). In the remaining 5 patients, diagnosis was made by fluid analysis from percutaneous cholecystostomy (n = 1, 1.3%), computerized tomography (CT) scan guided needle biopsy (n = 1, 1.3%) and autopsy (n = 3, 4.0%). Staging of the cancer was

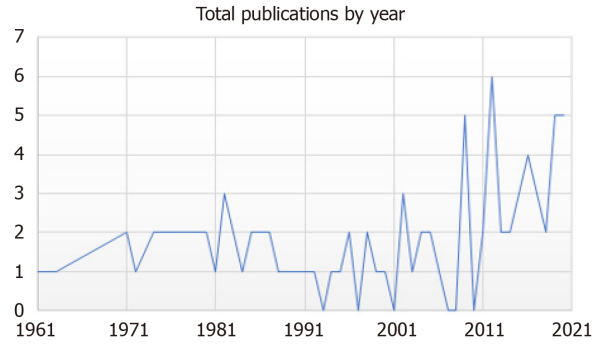


Figure 1 Paucity of gallbladder carcinosarcoma reports and trends by decade.

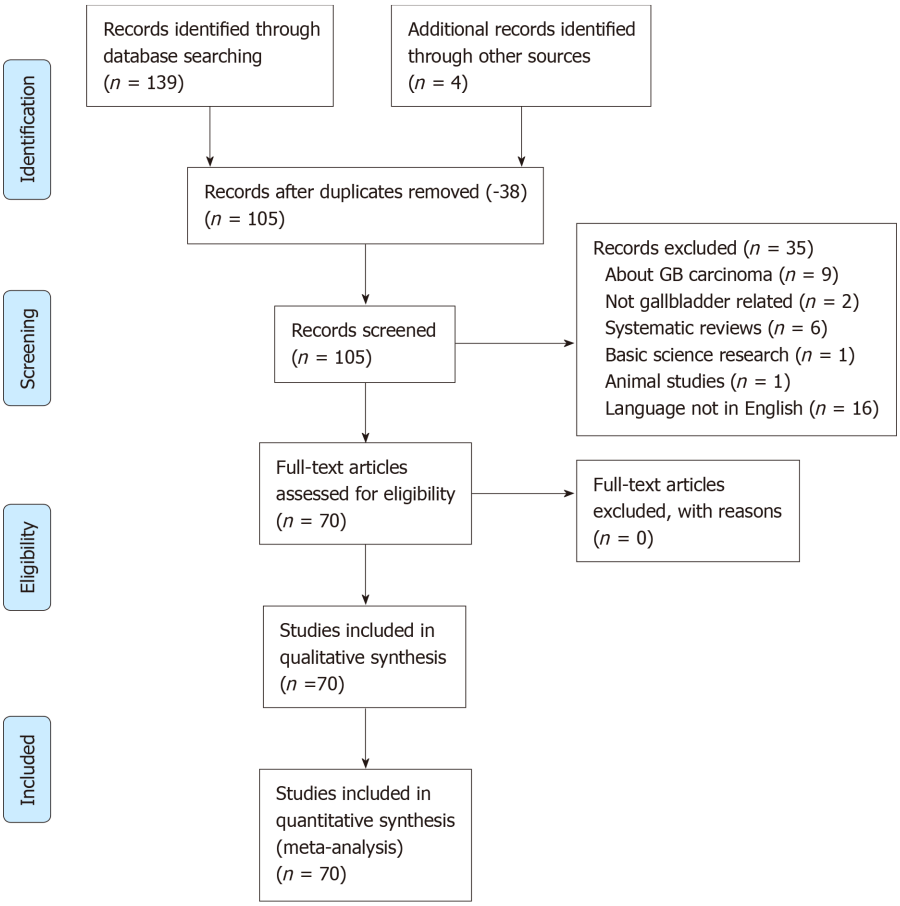


Figure 2 PRISMA diagram of articles searched on gallbladder carcinosarcoma. GB: Gallbladder

reported infrequently, with TNM system being the most common (n = 15, 19.7%). The majority of patients had stage II (n = 6, 40.0%) and stage III disease (n = 5, 33.3%). Three patients had stage IV disease (20.0%), and 1 patient had stage I disease (6.67%). Immunohistochemical stains (vimentin for mesenchymal components and cytokeratin for epithelial components) were reported in 50 patients (68.5%). Vimentin (n = 42, 84.0%), cytokeratin (n = 39, 78.0%) and Ki-67 staining (n = 7, 14.0%) were variably positive.

Fourteen patients (18.4%) received adjuvant chemotherapy. Various chemotherapy combinations included: gemcitabine and cisplatin, leucovorin and 5-fluorouracil (5-FU), cisplatin and doxorubicin, cisplatin and 5-FU, tegafur-uracil and gemcitabine and oxaliplatin and 5-FU. Palliative treatment was chosen in 4 patients (5.26%). Amongst all those reported, 32 patients contained both survival and tumor size data. Kaplan-Meier survival analysis was performed (Figure 3), and there was no significant difference in survival times (P = 0.301) for patients with tumors less than 5 cm in diameter compared to those with larger tumors.

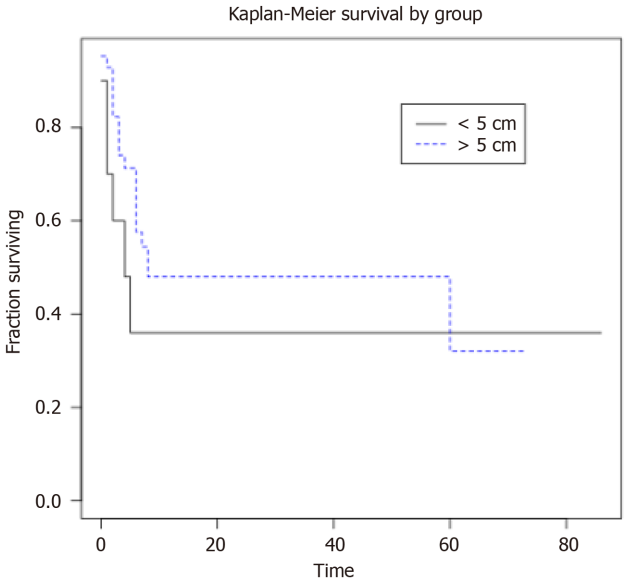


Figure 3 Kaplan-Meier survival curve measuring the difference in survival between patients with gallbladder carcinosarcoma of less than 5 cm diameter and more than 5 cm diameter (*P* = 0.301).

DISCUSSION

Gallbladder cancer is a rare neoplasm, accounting for about 0.5% of all gastrointestinal malignancies[73]. Most common gallbladder cancer is adenocarcinoma. GBCS is a rare form of gallbladder cancer, with only 78 cases reported. GBCS is characterized by carcinomatous and sarcomatous components and is made up of both epithelial and mesenchymal components. Commonly, the epithelial component consists of adenocarcinoma followed by the less common squamous cell carcinoma[74]. While there are multiple theories to justify the mixture of the epithelial and mesenchymal components, there is no consensus on the pathophysiology of the neoplasm. GBCS is considered the most aggressive biliary tract malignancy, usually discovered at late stages, and has poor prognosis[3].

Incidence

In terms of patient demographics, our results are consistent with the report of Zhang *et al*[3]. In a report including 68 GBCS patients, those authors reported a median age of 68 years (range: 45 to 91 years) with female predominance (female:male = 2.7:1), consistent with our results with a gender ratio of 2.32 and a mean age of 66.0 years (range: 40-91 years). Female preponderance is likely due to increased prevalence of gallstones in females. Zhang *et al*[3] noted gallstones in 66.7% of their patients. In our study, the incidence of gallstones was high (83%). However, gallstone presence was not specific nor sensitive in the diagnosis of GBCS, as not only are they a common finding in cancers of the gallbladder, only 1%-5% of patients with gallstones develop gallbladder malignancies. In our analysis of the literature, gallstone presence was only noted in 83.3% of patients where the presence of gallstones was assessed.

APBJ is also another risk factor of gallbladder malignancy[12]. Matsubayashi *et al*[12] reported a 72-year-old female patient with symptoms of abdominal pain. Laboratory investigations revealed raised alkaline phosphatase and gamma-glutamyl transpeptidase. CT scan confirmed a polypoid gallbladder mass. Magnetic resonance cholangiopancreatography scan showed ABPJ, and this was confirmed at subsequent endoscopic retrograde cholangiopancreatography. While APBJ is a well-known risk factor for gallbladder cancers[75], this was the first case of APBJ in GBCS noted in the literature.

Other risk factors mentioned include chronic cholecystitis, which could be both a risk factor and the manifestation of gallbladder malignancy. Unique to the gallbladder is a cycle of gallbladder epithelium damage and repair, enabling a chronic inflammatory environment from chronic cholecystitis[76]. This cycle of inflammation, injury, repair and regeneration increases cell turnover and oxidative stress. Yildiz *et al*[77] stated biliary tract to be the "consummate example of inflammation-associated carcinoma". Chronic inflammation from gallstone disease can lead to protein damage,

genetic mutations, inhibition of apoptosis, promotion of angiogenesis, modulation of cell adhesion and motility as well as immunosuppression. Chronic cholecystitis leads to gallbladder wall thickening, and CT or magnetic resonance imaging (MRI) scans are sensitive to detect wall thickness. However, it is not possible to distinguish if thickening of the gallbladder wall is due to inflammation or malignancy[78]. Thus, multidisciplinary discussion involving experienced radiologists and hepatobiliary surgical team is essential to make management plans for patients with suspicious gallbladder lesions.

Signs and symptoms

Clinical manifestations of GBCS are nonspecific, with symptoms such as abdominal pain localized to the right upper quadrant, constitutional symptoms, nausea, vomiting and fever. The mechanism resulting in constitutional symptoms in patients with cancer is multifactorial and not yet fully understood. It is thought that multiple pathways involving pro-cachectic and pro-inflammatory signals from tumor cells along with systemic inflammation of the host combine with widespread metabolic changes contribute to the manifestations of symptoms like anorexia and cachexia[79]. In particular, cholecystokinin is an integral peptide involved in satiety and regulating diet intake[80]. Given its role in gallbladder contraction, dysregulation of cholecystokinin could be involved in the manifestation of constitutional symptoms of anorexia in patients with GBCS.

Similarly, the pathophysiology of febrile response in malignancies is complex. Released pyrogenic cytokines from tumor cells and tissue macrophages induces a chain of events that result in reset of hypothalamic thermostat due to prostaglandin E2 and related pathways[81]. On physical examination, the presence of a right hypochondria tenderness or mass is not specific, and it does not rule out malignancy. Thus, if a patient is managed for suspected acute or chronic cholecystitis, a follow-up physical examination and imaging needs to be arranged to document resolution of inflammatory process. In this review, 2 asymptomatic patients were diagnosed with GBCS. From our analysis, Ishida *et al*[36] and Akatsu *et al*[46] reported incidental findings of GBCS on imaging findings for unrelated issues. Ishida *et al*[36] reported a 62-year-old female with unexpected calcification in the right upper abdomen in a CT meant for follow-up of percutaneous pinning of a left calcaneal fracture. Akatsu *et al*[46] reported a 76-year-old female who was on regular follow-up for cholelithiasis. Abdominal ultrasound revealed a heterogeneously hypoechoic mass around the gallbladder bed. In both patients, a preoperative diagnosis of possible gallbladder malignancy was made, and surgical exploration with subsequent cholecystectomy was performed.

Biochemical investigations

Biochemical abnormalities in GBCS are also mostly nonspecific. The most common derangements were transaminitis, hyperbilirubinemia and anemia. This was consistent with Ayoub *et al*[5] who reported that hepatic and inflammatory markers were often normal.

Presurgical diagnosis of gallbladder malignancies is difficult due to its varying presentations. Differentials to consider for such lesions when calcification is present include calcified gallstones, porcelain gallbladder and GBCS[78]. Our analysis noted cases where GBCS was initially diagnosed with cholelithiasis, acute cholecystitis, gallbladder empyema, diffuse peritonitis, pancreatic cancer and pyogenic liver abscess.

Imaging

As there are no radiological signs identified in the current literature that distinguishes GBCS from other gallbladder malignancies[50,51], the diagnosis is difficult even with imaging. For instance, Appelman *et al*[72] described a 91-year-old male presenting with yellow sclera, pale stools, dark urine and pruritus. His liver function tests were deranged with obstructive pattern, and a diagnosis of pancreatic cancer with biliary tract obstruction was made. The patient refused surgical intervention and died within 2 wk. Autopsy confirmed the diagnosis of metastatic disease with GBCS primary.

Khurram *et al*[4] reported a 64-year-old lady presented with right upper quadrant mass, intermittent fever and abdominal distension following a recent travel history to Ghana. CT scan revealed a hepatic lesion with coexisting gallbladder distension consistent with pyogenic liver abscess. Due to failure to respond to intravenous antibiotics, MRI scan was done. MRI scan showed a gallbladder fundus soft tissue lesion with local invasion into the liver[22]. Histopathological diagnosis of GBCS was

made after surgical excision. Hence, in the absence of a confirmatory preoperative diagnosis, all suspicious gallbladder lesions must be reviewed at multidisciplinary meetings.

Porcelain gallbladder, gallbladder tuberculosis and xanthogranulomatous cholecystitis are common benign conditions that can be confused with malignancy. Porcelain gallbladder is described as a hyperechoic focus with posterior acoustic shadowing on an ultrasound scan[82]. Ultrasound scan is not sensitive for regional and distant spread of malignancy. CT and MRI scans are more sensitive to detect contiguous spread to liver, regional lymph node involvement and distant metastases. Diffuse nodular thickening without layering, early enhancement, low apparent diffusion coefficient and high lesion to spinal cord ratio are MRI features suggestive of gallbladder cancer[83]. In addition, CT and MRI scans provide details that assist in surgical planning. 18-fluorodeoxyglucose-positron emission tomography-CT can aid in distinguishing between benign and malignant gallbladder lesions. Malignant lesions have high standardized uptake value. In a study reporting 30 patients with a mean age of 48.22 ± 31.33 years and gallbladder wall thickening (focal > 4 mm and diffuse > 7 mm), Gupta *et al*[84] reported that 18-fluorodeoxyglucose-positron emission tomography had high overall sensitivity (91%), specificity (79%), positive predictive value (77%), negative predictive value (92%) and diagnostic accuracy (84%).

Histological diagnosis

Diagnosis of GBCS is usually made after pathological analysis of a surgical specimen. In patients with unresectable neoplasms, tissue diagnosis can be achieved by percutaneous biopsy. This is essential to plan definitive chemotherapy[85]. In clearly resectable lesions, the role of percutaneous biopsy is debated due to risk of needle-tract seeding[86]. Furthermore, as the gallbladder is a hollow organ, bile spill and peritonitis remain a risk too[87]. As GBCS are rare tumors with poor prognostic outcomes, treatment options are not well defined, with little evidence supporting or refuting any postoperative adjuvant therapy. Okabayashi *et al*[88] and Mochizuki *et al*[8] both corroborate that surgical treatment remains the only cure for GBCS. While the histopathological features between GBCS and adenocarcinoma of the gallbladder are different, management is similar.

Surgical management

Currently, the consensus for treatment involves surgical excision of the gallbladder and extrahepatic bile duct, regional lymphadenectomy and even a pancreaticoduodenectomy depending on the extent of the growth[88]. Completion liver resection with or without lymphadenectomy and/or bile duct resection is an accepted standard for post simple cholecystectomy discovered gallbladder cancer with T_{1b} and higher stage. This approach not only involves two surgeries but also increases the risk of cutting through the tumor with potential for tumor seeding and dissemination. Yip *et al*[89] in a series of 40 patients with incidental gallbladder cancer reported that the majority of patients were not amenable for further curative resection. A report from Memorial Sloan-Kettering Cancer Centre involving 116 patients showed that survival of patients with residual disease was not different than survival of patients with stage IV disease, and neither group of patients benefit from reoperation[90]. Thus, single surgery may be better.

Radical cholecystectomy has higher morbidity as compared to simple cholecystectomy. Thus, the concept of something intermediate, *i.e.*, extended cholecystectomy, is attractive. Fujisaki *et al*[91] reported a case describing the concept of laparoscopic extended cholecystectomy with 1 cm liver margin; however, they proposed open conversion when intraoperative histology showed gallbladder cancer invading the subserosal layer. With current advancements, laparoscopic extended cholecystectomy was noted to have lesser intraoperative and postoperative complications than open extended cholecystectomy[92].

The key differences between a 'radical' and 'extended' cholecystectomy are restricting the liver parenchyma transection to the 2 cm wedge of liver tissue and performing regional lymphadenectomy and choledochectomy only in selected patients. Radical cholecystectomy can be done by open, laparoscopic or robot assisted approach, with comparable short-term outcomes[93]. Overall, more data is required to determine the safety and feasibility of minimal access techniques in gallbladder malignancies. Due to the absence of histological diagnosis, management of suspicious gallbladder lesions must be determined by local resources, surgeon experience and access to technology.

In a recent systematic review, Frountzas *et al*[94] reported that many patients with xanthogranulomatous cholecystitis were managed with complex procedures like wedge hepatic resection and bile duct excision with high open conversion rate (35.0%) at planned cholecystectomy. Intraoperative frozen section analysis is a useful adjunct in surgical planning. While intraoperative frozen tissue diagnosis is relatively reliable to determine whether lesions are benign or malignant, it does not reliably detail the depth of invasion of gallbladder malignancies[95]. Furthermore, the accuracy of intraoperative frozen tissue diagnosis for GBCS has yet to be determined due to paucity of scientific data.

Adjuvant treatment

The adjuvant treatment reduces recurrence risk and improves survival outcomes by eliminating or controlling the micrometastatic disease. A meta-analysis of retrospective studies including 6712 gallbladder cancer patients reported that lymph node positive patients enjoyed the survival benefit[96]. Few reported patients consider the use of UFT: tegafur/uracil, gemcitabine or a combination of tegafur/gimeracil/oteracil. The median survival of GBCS is 7.8 mo[10], and the addition of such regimes has not shown to improve survival[38]. There is a report by Pu *et al*[38] of using a combination of 5-FU (commonly used in gallbladder cancer) and oxaliplatin (commonly used in sarcomas). They reported a 59-year-old female coming in with right upper quadrant pain, fever and a raised CA19-9 level of 12000 U/mL, which was confirmed to be GBCS. The patient received oxaliplatin 150 mg and 5-FU 500 mg intravenously every 30 d for 6 cycles. At 6-mo follow-up, she did not reveal any signs of recurrence.

Adjuvant radiotherapy is shown to be of value in reducing local recurrence in selected patients with gallbladder cancer. In a study including 4180 patients with resected gallbladder cancer diagnosed from 1988 to 2003 from the Surveillance, Epidemiology, and End results database, Wang *et al*[97] reported that adjuvant radiotherapy provides survival benefit in node positive or T2 and higher stage disease. A single arm phase II study conducted by South West Oncology Group reported that gemcitabine plus capecitabine, followed by radiation (45 Gy to regional lymphatics, 54-59.4 Gy to tumor bed) and capecitabine resulted in 56% 2-year survival rate for patients with gallbladder cancer. Based on these results, the American Society of Clinical Oncology guidelines recommend chemotherapy plus radiation in gallbladder cancer patients with R1 resection[98]. There is no data to support neoadjuvant chemotherapy. Due to aggressive biological behavior, rapid progression or recurrence is common, and this is associated with a myriad of constitutional symptoms. For holistic care, management of the patients' subjective symptoms of anorexia and lethargy needs to be considered. Testosterone replacement therapy helps alleviate such symptoms in male patients with advanced cancer[99].

Prognosis

Generally, the prognosis of GBCS is poor. The majority of patients presenting to the hospital are locally advanced, with liver metastasis and peritoneal dissemination. Other metastasis sites reported include adrenal glands, pancreas, diaphragm and the lower thoracic vertebrae. Zhang *et al*[3] reported a mean survival time of 17.5 mo, with 1-year and 5-year survival rates at (19 ± 5)% and (16 ± 5)%, respectively. While it was previously noted the longest survival time to be reported as 54 mo by Uzun *et al*[44], our review noted 86 mo to be the longest survival time[10]. Aldossary *et al*[10] reported a 62-year-old female patient who complained of severe intermittent right upper quadrant pain of 2 mo duration. Laboratory investigations were normal, and ultrasound suggested a gallbladder with large stones and a non-mobile echogenic mass. A stage II (pT2, pN0, M0) moderately differentiated GBCS was noted on histology after laparoscopic cholecystectomy. The patient underwent 14 cycles of adjuvant chemotherapy. She had local recurrence at 2 years. Wide local excision of the mass with wedge resection of the liver, lymphadenectomy and partial gastrectomy was done. The patient remained disease free for 86 mo. Zhang *et al*[3] also claim that tumors smaller than 5 cm had a more prolonged survival, however we did not observe this. More data is required to confirm this, as only 28 patients detailing both the survival data and size of tumor have been reported.

Role of tumor markers

GBCS is not noted to have association with any tumor markers. Consistent with the current literature, most of the patients did not note any raised tumor markers[5]. However, it is still common practice for physicians to perform tumor marker levels

such as CA19-9, carcinoembryonic antigen and alpha-fetoprotein when considering possible differentials for masses in the gallbladder as well as for prognostication. For instance, Hayashi *et al*[100] propose that alpha-fetoprotein-producing carcinomas of the gallbladder are more likely to metastasize to the liver and have poor prognosis. CA19-9 is typically associated with pancreatobiliary malignancies but has a limited role in clinical practice[101]. Thus, prognostication is relied typically on histological features, pathologic stage as well as immunohistochemistry. Immunohistochemistry for the mesenchymal and epithelial components yield positive staining for vimentin and cytokeratin[45]. Our review shows that the majority of the patients had positive staining for vimentin (81.2%) and cytokeratin (79.2%). Additionally, Ki-67 was suggested by Kubota *et al*[45] to have prognostic value, whereby its presence signifies a possibly higher malignant proliferative potential for GBCS. However, this claim needs to be further investigated as Kubota *et al*[45] examined this immunohistochemical marker in only 1 patient with CSGB.

Comparison to gallbladder adenocarcinoma

There is substantial overlap of risk factors, diagnosis and treatment of GBCS with gallbladder adenocarcinoma. Thus, the majority of authors extrapolate the clinical characteristics of gallbladder adenocarcinoma to determine the best approach to diagnosis and management of GBCS. From this review, we can determine three key differences between GBCS and gallbladder adenocarcinoma. First, tumor markers have limited utility in patients with GBCS. In a study of 55 cases by Shukla *et al*[102], it is noted that the combination of CA-125 and CA19-9 helped detect gallbladder malignancy in patients with gallstones (80.7%). Second, the prognosis of GBCS may be marginally better compared to carcinoma of the gallbladder. In the meta-analysis by Zhang *et al*[3], it was noted that the survival rate was slightly better (16% ± 5% 5-year survival) compared to carcinoma of the gallbladder (0-10% 5-year survival). Thus, the identification of GBCS will be useful to determine the prognosis for patients albeit with only a small variation between the two. Third, immunohistochemistry markers like vimentin and cytokeratin are associated with diagnosis of GBCS.

CONCLUSION

In conclusion, GBCS is more common in females. Gallstones and chronic cholecystitis are risk factors for GBCS. Serum biochemistry and tumor markers have a limited role in diagnosis. Typical imaging modalities can assist to establish a diagnosis in patients with suspicious gallbladder lesions. Multiple imaging modalities are complementary. Multidisciplinary oncology board discussions are essential to guide management plans. Surgery is currently the only curative option for GBCS, and size of the tumor does not impact prognosis. While most features of GBCS parallel that of carcinomas of the gallbladder clinically, identification of GBCS specifically allows clinicians to determine overall prognosis. Due to paucity of reported cases, more evidence is required before meaningful and valid evidence-based patient-centric recommendations can be made. This review serves to educate and raise awareness among the clinicians dealing with gallbladder malignancies. It is likely that there are more clinical differences between GBCS and common forms of gallbladder cancer; active reporting of cases will help enhance understanding of this rare cancer.

ARTICLE HIGHLIGHTS

Research background

Literature on gallbladder carcinosarcoma (GBCS) is currently scarce, with less than 100 cases reported since the first case by Karl Lansteiner.

Research motivation

While there has been efforts by Zhang *et al* in 2008 to consolidate the literature, there has not been a review of the current literature since.

Research objectives

This study aims to fill this gap by providing a comprehensive overview of GBCS.

Research methods

A literature review was performed on the PubMed database using the keywords "Gallbladder" AND "Carcinosarcoma" from 1970 to 2021, where relevant articles were included. Animal studies, gallbladder carcinoma and non-gallbladder pathology as well as articles that were not in English or Japanese were excluded.

Research results

GBCS is more common in females. Gallstones and chronic cholecystitis are risk factors for GBCS. Serum biochemistry and tumor markers a have limited role in diagnosis. Typical imaging modalities can assist to establish a diagnosis in patients with suspicious gallbladder lesions. Multiple imaging modalities are complementary. Multidisciplinary oncology board discussions are essential to guide management plans. Surgery is currently the only curative option for GBCS, and size of the tumor does not impact prognosis.

Research conclusions

While most features of GBCS parallel that of carcinomas of the gallbladder clinically, identification of GBCS specifically allows clinicians to determine overall prognosis. Due to paucity of reported cases, more evidence is required before meaningful and valid evidence-based patient-centric recommendations can be made.

Research perspectives

Due to the paucity of the number of reported cases, more active reporting of such should be encouraged to further understand this malignancy.

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