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- 1 Growth hormone and gastrointestinal malignancy: An intriguing link

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Growth hormone and gastrointestinal malignancy: An intriguing link

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Abstract

Growth hormone (GH) excess is associated with several systemic complications, one of which is the increased risk of neoplastic processes particularly of the gastrointestinal (GI) tract. Among the GI neoplasms, the most reported association is with benign and malignant neoplasms of the colon. In the majority of published literature, an increased incidence of GI neoplasms, both colonic adenomas as well as colorectal carcinoma is reported. However, the studies on colon cancer-specific mortality rate are conflicting with recent studies reporting similar cancer-specific mortality rates in comparison to controls. Many studies have reported an association of colorectal neoplasms with GH levels. Pathogenic mechanisms put forward to explain this association of GH excess and GI neoplasms primarily involve the increased GH-insulin-like growth factor 1 (IGF-1) signaling. Both GH and IGF-1 have proliferative, anti-apoptotic, and angiogenic effects on the systemic tissues leading to cellular proliferation. Other contributing factors to the increased risk of GI neoplasms include slow intestinal transit with a redundant large bowel, altered bile acids, deranged local immune response, shared genetic susceptibility factors and hyperinsulinemia. In view of the increased risk association, most guidelines for the care of acromegaly patients recommend an initial screening colonoscopy. Recommendations for further follow-up colonoscopy differ but broadly, the guidelines agree that it depends on the findings at first colonoscopy and state of remission of GH excess. Regarding the concern about the risk of colorectal cancers in patients receiving recombinant GH therapy, most cohort studies do not show an increased risk.

Key Words: Acromegaly; Colonoscopy; Colorectal carcinoma; Recombinant growth

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Core Tip: Growth hormone (GH) excess is associated with systemic complications including an increased risk of gastrointestinal (GI) neoplasms. Although most studies show an increased incidence of all GI neoplasms, most evidence is for increased colorectal adenomas and carcinomas. However, recent studies show similar colon cancer-specific mortality rates in acromegaly as compared to the general population. Screening guidelines for acromegaly recommend an initial colonoscopy in all patients with further follow-up recommended for patients with high risk. Most cohort studies do not report any increased risk of GI neoplasms for patients on recombinant GH therapy.

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INTRODUCTION

Physiologically, growth hormone (GH) is secreted from the anterior pituitary gland and produces its tissue mediator, insulin-like growth factor 1 (IGF-1) from the liver. The GH-IGF-1 duo have potential proliferative, anti-apoptotic, and angiogenetic effects on the systemic tissues. Acromegaly is a rare endocrine disorder of excessive GH production mostly from a pituitary adenoma[1]. As the disease is indolent and evolves very slowly, most of the patients suffer a significant diagnostic delay[2]. Due to this prolonged exposure to GH-IGF-1 excess, patients with acromegaly suffer from metabolic as well as systemic complications involving musculoskeletal, cardiovascular, respiratory, neurological and gastrointestinal (GI) systems[3]. Epidemiological data also suggest a higher prevalence of neoplastic complications in patients with acromegaly because of the proliferative action of GH and IGF-1[4]. Among the various neoplastic complications in acromegaly, tumors involving the GI system are well known.

The purpose of this review was to explore the various GI neoplasms reported in acromegaly as well as the pathophysiology behind it. The current updates regarding the screening of GI neoplasms in acromegaly have been discussed. The potential risk of GI neoplasia associated with therapeutic GH administration has also been reviewed.

EPIDEMIOLOGY OF GI NEOPLASIA IN GH EXCESS

Among the GI neoplasms reportedly associated with GH excess (acromegaly), colorectal neoplasms are one of the most common. Apart from malignant neoplasms, the incidence of various benign neoplasms such as polyps are also reportedly associated with GH excess. In the following sections, the major studies that reported the risk of benign as well as malignant neoplasms of the GI tract are described.

Malignant colorectal neoplasm

The increased risk of colorectal neoplasm in acromegaly was initially reported in the studies by Klein *et al*[5] and Ituarte *et al*[6]. Later, the majority of the published studies in patients with acromegaly reported higher incidences of colorectal neoplasms. In a nationwide population-based study from Sweden, a total of 1296 patients with acromegaly were included[7]. The incidence of colorectal and anal cancer was significantly increased in the acromegaly cohort (standardized incidence ratio [SIR] 1.5; 95% confidence interval [CI]: 1.0 to 2.2). Of the 28 reported cases of colorectal and anal carcinomas, two were neuroendocrine tumors. The incidence of benign colorectal and anal tumors was also significantly high in patients with acromegaly (SIR 2.3; 95%CI: 1.6 to 3.3). In another recent study from Argentina, the risk of colorectal neoplasms was compared between 70 patients with acromegaly and control population[8]. Although there was no significant increase in the risk of non-precancerous polyp ($P = 0.69$) in patients with acromegaly, the risk of both advanced adenoma ($P = 0.0006$) and colorectal carcinoma ($P = 0.0063$) was significantly high. The increased risk of colorectal cancer (SIR 1.67; 95%CI: 1.07 to 2.58) was also reported in an Italian multicenter cohort study of 1512 patients with acromegaly[9]. In the subgroup analysis, the increased risk of colorectal cancer was found in female patients but not in male patients. In the largest study from the United States, cancer incidence was studied in 1041 men with acromegaly

[10]. The cancer risk of the overall digestive organ was increased (SIR 2.0; 95%CI: 1.3 to 2.9) including the colon (SIR 3.1; 95%CI: 1.7 to 5.1) in comparison to the control population. In a combined cohort from Sweden and Denmark, a total of 1634 patients with acromegaly were followed up for 10.3 years[11]. The risk of overall digestive organ cancers (SIR 2.1; 95%CI: 1.6 to 2.7) as well as colon (SIR 2.6; 95%CI: 1.6 to 3.8) and rectal cancers (SIR 2.5; 95%CI: 1.3 to 4.2) was increased. In a single center study of 140 patients with active acromegaly from Japan, the risk of colon cancer was reported to be as high as 17.4 (SIR) in females and 19.0 (SIR) in males[12]. In another small study of 19 patients with acromegaly from Japan, the authors reported a higher risk of colon cancer (odds ratio [OR] 9.8; 95%CI: 1.0 to 97.2) and colon adenoma (OR 4.2; 95%CI: 1.3 to 13.7) compared to the matched control population[13]. The risk of colon cancer was found to be significantly associated with high GH levels in this study. In a multicenter study from Italy, 233 patients of acromegaly were evaluated for the risk of colonic neoplasia[14]. The authors reported an increased risk of colon carcinoma (risk ratio [RR] 4.9; 95%CI: 1.1 to 22.4) in this study.

However, not all the studies had reported significantly increased risk of colorectal neoplasms in acromegaly patients. The risk of colorectal cancer was not significantly elevated (SIR 1.4; 95%CI: 0.7 to 2.6) in a cohort of 529 Danish acromegaly patients[15]. Similarly, the incidence of colorectal cancer was not significantly higher in the cohort of acromegaly patients from Poland and Germany[16,17]. In the nationwide cohort of Finnish acromegaly patients, the authors did not report any excess risk of colorectal cancer (SIR 1.9; 95%CI: 0.7 to 4.1)[18]. However, a trend of increased incidence of colorectal cancer (SIR 4.44, 95%CI: 0.91 to 13.0) was observed in acromegaly patients who responded poorly with treatment (posttreatment GH > 2.5 µg/L). In one of the largest cohorts of acromegaly patients from the United Kingdom, a non-significant trend of increased risk of colon cancer (SIR 1.67; 95%CI: 0.87 to 2.93; $P = 0.06$) was reported[19]. However, no significant difference in the risk of rectal cancer (SIR 0.86; 95%CI: 0.23 to 2.20; $P = 0.69$) had been reported in this study. The results of the major studies have been summarized in Table 1.

In the first meta-analysis by Renehan *et al*[20], which included only population-based studies, increased risk of colorectal cancer (RR 2.04; 95%CI: 1.32 to 3.14) in acromegaly was reported. In another meta-analysis that evaluated the risk of colorectal neoplasm in acromegaly, a total of 701 patients from nine studies was included[21]. The risk of colonic adenoma (OR 2.486; CI: 1.908 to 3.238) as well as colorectal carcinoma (OR 4.351; CI: 1.533 to 12.354) were found to be significantly higher in patients with acromegaly. In the most recent study by Dal *et al*[15], the authors evaluated cancer risk in patients of Danish acromegaly cohort as well as performed a meta-analysis to determine the cancer risk in patients with acromegaly. In this meta-analysis, a total of 9677 acromegaly patients from 23 studies were included. Among the cancers related with the GI tract, the risk of colorectal carcinoma (SIR 2.6; 95%CI: 1.7 to 4.0; 14 studies) and gastric carcinoma (SIR 2.0; 95%CI: 1.4 to 2.9) was found to be significantly increased in acromegaly patients. The risk of colorectal carcinomas was found to be elevated unequivocally in single center (SIR 7.3; 95%CI: 2.6 to 20.6), multicenter (SIR 2.0; 95%CI: 1.3 to 3.1) as well as in population based (SIR 2.2; 95%CI: 1.7 to 3.0) studies.

There is no consensus on whether the rate of colon cancer-specific mortality is increased in acromegaly. While the initial studies demonstrated an increased cancer-specific mortality rate, later studies did not corroborate these findings[10,22]. The study by Ritvonen *et al*[22] showed that although the overall standardized mortality rate was increased in acromegaly patients as compared to controls, cancer-specific mortality rate was similar. The overall cancer-related mortality due to GI cancer was not significantly increased (standardized mortality rate 1.3; 95%CI: 0.8-1.9) in the nationwide population-based study of acromegaly patients from Sweden[7].

Other malignant GI neoplasms

Studies that reported an increased risk of GI neoplasms other than those of colorectal origin in acromegaly are sparse in the literature. In the nationwide study from Sweden, the risk of gastric carcinoma (SIR 1.7; 95%CI: 0.6 to 4.0; $P = 0.35$) was not significantly higher in acromegaly patients[7]. A trend toward increased risk of gastric carcinoma (4 patients in acromegaly cohort *vs* 1.1 expected from population-based registry; SIR not calculated) in acromegaly patients was reported in the Danish acromegaly cohort[15]. Ron *et al*[10] had reported increased risk of gastric cancer (SIR 2.5; 95%CI: 0.8 to 6.0) in a cohort of acromegaly patients from the United States. The increased risk of pancreatic cancer (SIR 2.6; 95%CI: 1.0 to 5.3) was reported in a cohort of acromegaly patients from Sweden (subgroup analysis)[11]. Similarly, in the study from Sweden, a trend of increased risk of pancreatic cancer (SIR 2.1; 95%CI: 0.9 to 4.1; $P = 0.082$) has also been reported[7]. In the combined cohort of acromegaly patients from Sweden and Denmark, increased risk of small intestine cancer (SIR 6.0; 95%CI: 1.2 to 17.4) was reported[11]. The risk of esophageal cancer (SIR 3.1; 95%CI: 1.3 to 6.0) was also found to be elevated in acromegaly patients[10].

Benign GI neoplasms

Apart from increased risk of malignancy, a higher incidence of GI polyps was also reported in acromegaly patients. A high prevalence of colonic polyp (as high as 40%) has been reported in cohorts of acromegaly patients[12,23,24]. In the study by Gonzalez *et al*[25], a total of 165 acromegaly patients from Mexico were included who were evaluated by colonoscopy. The authors reported significantly increased risk of development of colonic polyps overall (RR 6.21; 95%CI: 4.04 to 9.48; $P < 0.001$) as well

Table 1 The risk of colorectal and gastrointestinal neoplasms in acromegaly: The major studies

Ref.	Country	Colorectal cancer risk	Other GI cancer risk
Esposito <i>et al</i> [7], 2021	Sweden	SIR 1.5; 95%CI: 1.0 to 2.2	Stomach (SIR 1.7; 95%CI: 0.6 to 4.0); pancreas (SIR 2.1; 95%CI: 0.9 to 4.1)
Battistone <i>et al</i> [8], 2021	Argentina	5.7% <i>vs</i> 0% ($P = 0.0063$)	Advanced colorectal adenoma: 27.3% <i>vs</i> 7% ($P = 0.0006$)
Terzolo <i>et al</i> [9], 2017	Italy	SIR 1.67; 95%CI: 1.07 to 2.58	NA
Dal <i>et al</i> [15], 2018	Denmark	SIR 1.4; 95%CI: 0.7 to 2.6	NA
Wolinski <i>et al</i> [16], 2017	Poland	2% <i>vs</i> 0% ($P = 0.14$)	NA
Petroff <i>et al</i> [17], 2015	Germany	4 <i>vs</i> 6.6 ($P = 0.43$)	NA
Kauppien-Makelin <i>et al</i> [18], 2010	Finland	SIR 1.9; 95%CI: 0.7 to 4.1	NA
Kurimoto <i>et al</i> [12], 2008	Japan	Male: (SIR 19.0; 95%CI: 5.18 to 48.64); female: (SIR 17.4; 95%CI: 4.74 to 44.55)	NA
Matano <i>et al</i> [13], 2005	Japan	OR 9.8; 95%CI: 1.0 to 97.2	NA
Terzolo <i>et al</i> [14], 2005	Italy	RR 4.9 (95%CI: 1.1 to 22.4).	NA
Baris <i>et al</i> [11], 2002	Sweden and Denmark	Colon (SIR 2.6; 95%CI: 1.6 to 3.8); rectum (SIR 2.5; 95%CI: 1.3 to 4.2)	Overall digestive system (SIR 2.1; 95%CI: 1.6 to 2.7); small intestine (SIR 6.0; 95%CI: 1.2 to 17.4)
Orme <i>et al</i> [19], 1998	United Kingdom	Colon cancer (SIR 1.67; 95%CI: 0.87 to 2.93, $P = 0.06$); rectal cancer (SIR 0.86; 95%CI: 0.23 to 2.20, $P = 0.69$)	NA
Ron <i>et al</i> [10], 1991	United States	Colon (SIR 3.1; 95%CI: 1.7 to 5.1)	Overall digestive system (SIR 2.0; 95%CI: 1.3 to 2.9); esophagus (SIR 3.1; 95%CI: 1.3 to 6.0); stomach (SIR 2.5; 95%CI: 0.8 to 6.0)

CI: Confidence interval; GI: Gastrointestinal; NA: Not available; OR: Odds ratio; RR: Risk ratio; SIR: Standardized incidence rate.

as both adenomatous (RR 4.86; 95%CI: 2.5 to 9.92; $P < 0.001$) and non-adenomatous colonic polyps (RR 4.85; 95%CI: 2.18-8.81; $P < 0.001$). Majority of the polyps were located in descending colon and recto-sigmoid colon. High serum IGF-1 level was significantly associated with development of colonic polyps. In a study from Turkey, an increased risk of colonic polyps overall (OR 2.04, 95%CI: 1.2 to 3.47; $P = 0.012$) and hyperplastic colonic polyps (OR 3.04, 95%CI: 1.4 to 6.6) were reported in acromegaly patients [26]. Higher IGF-1, male sex, and older age were associated with an increased risk of polyp. However, the prevalence of adenomatous polyp was not significantly different in acromegaly patients in comparison to control population. In another study from Turkey, where 66 acromegaly patients were compared with matched controls, a significantly higher risk of colorectal polyps was reported in acromegaly patients (OR: 3.191, 95%CI: 1.25 to 8.13)[27]. In these patients, 72% of the polyps were found at the rectosigmoid colon. In another recent study from Japan, which evaluated 178 acromegaly patients, significantly higher colorectal polyps were detected in patients with acromegaly compared to controls (66.8% *vs* 24.2%; $P < 0.001$)[28]. Moreover, the polyps were larger in size and mostly located in rectosigmoid colon in acromegaly patients. Agarwal *et al*[29] had also reported increased risk of colonic polyp (10.6% *vs* 0.8%) in a cohort of 47 Indian acromegaly patients. In the study by Matano *et al*[13], a high risk of hyperplastic polyps (OR 8.3; 95%CI: 2.1 to 3.27) in acromegaly patients has been reported. The authors also reported a possible association with high GH levels. In a multicenter study from Italy, Terzolo *et al*[14] also reported elevated risk of benign adenomatous polyps (RR 1.7; 95%CI: 1.1 to 2.5) in acromegaly patients. In the meta-analysis by Rokkas *et al*[21], a total of 573 acromegaly patients from seven studies were included to evaluate the risk of colonic polyps. The authors reported higher risk of hyperplastic polyp (OR 3.557; 95%CI: 2.587 to 4.891) in the pooled analysis.

Increased prevalence of small bowel polyps has also been reported in a study from Mexico, which used capsule endoscopy for detection of polyps in 61 acromegaly patients[30]. All of the detected polyps in acromegaly patients were pedunculated and located in the jejunum. Ronchi *et al*[31] also evaluated the risk of small bowel polyps in 18 acromegaly patients using the video capsule endoscopy technique. The prevalence of small bowel polyps was found to be significantly higher (RR 2.50; 95%CI: 1.23 to 5.07). However, there was no significant difference in overall small bowel lesions between the acromegaly and control population. Apart from small bowel polyps, the incidence of gall bladder and gastric polyps was also reported in acromegaly patients[12]. In a retrospective study, increased prevalence of gall bladder

polyps (29.03% *vs* 4.62%, RR 6.29; 95%CI: 3.62 to 10.96; $P < 0.01$) were reported in a cohort of 31 newly diagnosed acromegaly patients from the United Kingdom[32]. High GH levels and older age (> 50 years) were associated with a higher risk of gall bladder polyps in these patients.

PATHOGENESIS OF GI NEOPLASMS IN ACROMEGALY

Colon cancer

Several hypotheses have been put forward regarding the pathogenic mechanisms underlying the association between acromegaly and increased risk of GI malignancy particularly colon cancer[20]. Of these, the primary mechanism involves the GH-IGF-1 axis. Other contributing factors include slow intestinal transit with a redundant large bowel, altered bile acids, deranged local immune response, shared genetic susceptibility factors and hyperinsulinemia[20,33].

Excess GH signaling: GH acts on most tissues through the GH receptor (GHR) and is proliferative and anti-apoptotic in its action[34]. The human colonic epithelia has been shown to harbor GHR abundantly [35]. Excess GH acts through the GHR on the colonic epithelia and activates the Janus kinase-signal transducer and activator of transcription 5 (STAT5) pathway[34]. STAT5 exerts cell proliferative and anti-apoptotic activity and is associated with malignancy in tissues other than the colon as well, such as the breast and the prostate[36,37]. In human colonic adenocarcinoma, the increased cellular expression of STAT5 has been found and correlates with a poor prognosis[38,39]. These studies demonstrate the role of GH-STAT5 signaling in colon cancer independent of IGF-1 signaling.

Excess IGF-1 signaling: GH mediates somatic growth predominantly by inducing the production of IGF-1 from the liver. Excess IGF-1 seen in acromegaly affects cellular growth and apoptosis at various tissue levels with the net result of favoring tumor progression[40,41]. Similar to GHR, IGF-1 receptors (IGF-1Rs) are expressed in human colonic adenomas and carcinomas[42]. While some studies report no difference in IGF-1R expression in colon cancer compared to normal colonic mucosa, others found an overexpression of IGF-1R[43-45]. Cats *et al*[46] found increased colonic cell proliferation measured by a labeling index in patients with acromegaly and it correlated with IGF-1 levels. However, in contrast to clear evidence of excessive local IGF-1 signaling, studies demonstrating an association between serum IGF-1 levels and the presence of colonic neoplasia is conflicting[25,47,48].

Increased IGFBP-3 and IGF-2 levels: The levels of IGF-binding protein 3 (IGFBP-3) are increased in acromegaly. IGFBP-3 has anti-proliferative and pro-apoptotic properties making it tumor-protective. In acromegaly, however, elevated GH increases the IGF-1 to IGFBP-3 ratio tipping in favor of increased cancer risk[49]. A study also found increased expression of IGF-2 transcripts in colorectal carcinoma cell lines suggesting that the growth of colorectal cancer could be mediated by IGF-2-mediated autocrine action on IGF-1R[50].

Other factors: Altered local immunity characterized by decrease in B cells, natural killer cells, and helper-inducer T cells has been associated with sporadic colon carcinogenesis and is also found in colonic mucosa of acromegaly patients[20]. Hyperinsulinemia as an underlying factor has been hypothesized to be associated with increased risk of colon cancer as the excess insulin acts through both insulin as well as IGF-1 receptors present abundantly on the colonic mucosa[51]. Acromegaly and type 2 diabetes mellitus are both associated with hyperinsulinemia and increased risk of colon cancer making hyperinsulinemia a plausible common underlying factor[49]. Few studies exist that have explored the genetic predisposition for colon cancer in patients with acromegaly. A study evaluated the polymorphisms in C677T methylene-tetrahydrofolate reductase gene and found that the TT genotype is associated with the increased odds of colon cancer in patients with acromegaly[52]. Association of some factors such as increased bile acids and slower colon transit time with risk of colon cancer in acromegaly are inconclusive[20].

To summarize, evidence for the pathogenic basis for increased risk of colon cancer in acromegaly is most robust for the GH-IGF-1 axis. Other contributing factors such as hyperinsulinemia, genetic predisposition, bile acids, and slower colon transit time need more evidence to substantiate the risk association (Figure 1).

Colon polyps

The French Acromegaly registry found that serum IGF-1 levels, but not GH levels, correlated with the presence of colorectal polyps even after adjustment for age, body mass index, and smoking[53]. Similar conclusion associating the occurrence of colonic polyps with markers of disease activity, especially serum IGF 1 levels, have been found in other studies as well[54,55]. The study by Parolin *et al*[55] also demonstrated an association between increased polyps size and untreated acromegaly.

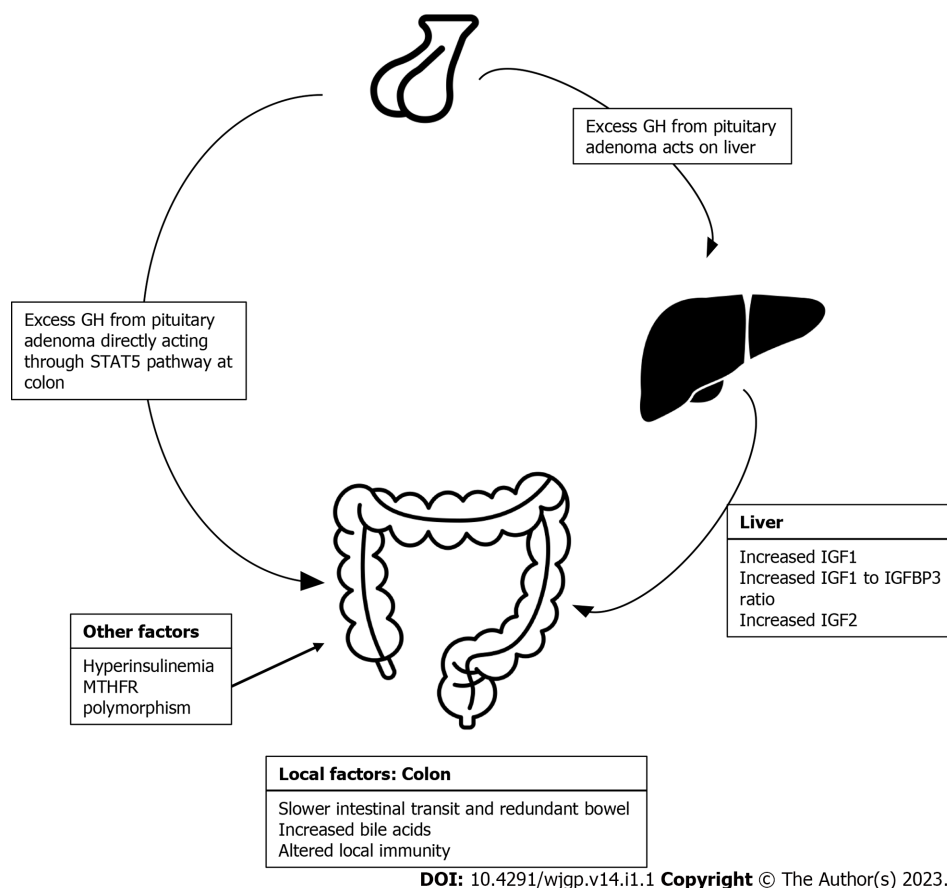


Figure 1 Pathogenesis of colorectal neoplasm in acromegaly. GH: Growth hormone; STAT5: Signal transducer and activator of transcription 5; IGF: Insulin-like growth factor; IGFBP-3: Insulin-like growth factor binding protein 3; MTHFR: Methylene-tetrahydrofolate reductase.

SCREENING MODALITIES FOR COLORECTAL LESIONS IN ACROMEGALY

There is a good agreement in the published literature that the prevalence and incidence of colonic lesions is increased in patients with acromegaly. Therefore, most guidelines recommend an initial screening colonoscopy for all patients with acromegaly. Melmed *et al*[56] in the guidelines for patients with acromegaly in 2002 recommended that colonoscopy should be performed every 3-5 years depending upon the family history and previous polyps detection. Their 2009 update recommended an initial colonoscopy in all patients with acromegaly followed by subsequent follow-up similar to the general population[57]. The Endocrine Society clinical practice guidelines for acromegaly in 2014 recommended a baseline screening colonoscopy in all patients at the time of diagnosis while a repeat colonoscopy is suggested once every 5 years in those with a polyp or elevated IGF-1 levels and once every 10 years in those without a polyp and normal IGF-1 levels. On similar lines, the British Society of Gastroenterology and Association of Coloproctology considered acromegaly as a moderate to high-risk group for colorectal cancer and suggested in their 2010 guideline update that patients with acromegaly should be offered regular screening with colonoscopy beginning at the age of 40 years. They recommended that the frequency of repeat colonoscopy be determined by the findings at the initial colonoscopy and the activity of acromegaly. Patients with adenoma at first screening or elevated serum IGF-1 levels should be screened with a repeat colonoscopy 3-yearly, while those with normal initial colonoscopy or normal GH/IGF-1 levels should be screened every 5-10 years[58].

Although the above-mentioned guidelines recommend a more frequent follow-up colonoscopy in acromegaly patients, evidence accumulating in the last decade do not entirely support this strategy. Recent data do not show an increased colon cancer-specific mortality in acromegaly patients, especially in those with controlled disease[19,59]. A recent meta-analysis concluded that although cancer-specific mortality rate in acromegaly was increased in uncontrolled disease, it was similar to the general population in the subgroup with controlled disease. They concluded that the mortality rates decreased in the last decade in patients with acromegaly and the cancer incidence was more related to the longer age of survival rather than the GH/IGF-1 axis[60]. The presence of a colonic adenoma at first colonoscopy also predicted the occurrence of an adenoma at second (OR 4.4, 95%CI: 1.9 to 10.4) or third colonoscopies (OR 8.8, 95%CI: 2.9 to 26.5%)[54]. More importantly, a normal first colonoscopy in acromegaly patients was associated with normal second and third colonoscopies[54]. The above study findings highlight that the presence of a colonic lesion at first colonoscopy coupled with uncontrolled or

untreated acromegaly with high serum IGF-1 levels are associated with the greatest risk of incident colonic lesion. In view of the recent evidence, although both the Acromegaly Consensus Group and the Pituitary Society, recommend an initial screening colonoscopy in patients with acromegaly, they toned down their recommendation to suggest that follow-up colonoscopy testing should be performed similar to that in the general population[61,62].

Colonoscopy is technically difficult in acromegaly due to the increased length of colon and it requires a better and longer preparation time[33]. In addition, a pan-colonoscopy rather than a sigmoidoscopy is essential in acromegaly, since one-quarter of the adenomas and one-half of the carcinomas occur in the ascending and transverse colon[21].

Fecal occult blood and computed tomography (CT) colonography are the other modalities that have been tried for the diagnosis of colorectal lesions in patients with acromegaly. In a study by Bogazzi *et al* [63], colonoscopy was superior to fecal occult blood testing in detecting colonic lesions in acromegaly. By contrast, CT colonography was found to be comparable to conventional colonoscopy in terms of diagnostic accuracy, although there is an increased risk of radiation exposure and it does not allow for intervention[64].

GI NEOPLASMS IN GH THERAPY

Several components of the GH-IGF-1 axis have been linked to the risk of carcinogenesis. As a result, there remains a safety concern for recombinant GH (rGH) therapy, specifically when it is being used in patients with history of a cancer or in patients without systemic GH deficiency. The possible risk of GI malignancy with GH therapy was first reported by Swerdlow *et al*[65]. In this cohort study from the United Kingdom, 1848 children and young adults who received rGH were followed up for the evaluation of cancer incidence and mortality. The incidence of colorectal cancer was high (SIR 7.9, 95%CI: 1.0 to 28.7; $P = 0.05$); however, the absolute number of patients who developed colorectal cancer was only 2. The risk of colorectal carcinoma has reportedly remained high (SIR 11.1, 95%CI: 1.3 to 39.9; $P = 0.03$) even after excluding the high-risk group (cancer survivor, chromosome fragility syndrome). The cancer mortality risk for colorectal cancer was also significantly high in this cohort. However, in the cohort study from France, the authors did not find any increased risk of colon cancer with rGH therapy [66]. In the prospective multinational Genetics and Neuroendocrinology of Short Stature International Study (GeNeSIS), more than 20000 patients who received rGH therapy were followed up. In this study also, the authors did not find any increased risk of cancer overall and only 1 patient developed rectal adenocarcinoma in the entire cohort[67]. In the recent Safety and Appropriateness of Growth Hormone Treatments in Europe cohort study, a total of 23984 patients who were treated with rGH in eight European countries (Belgium, France, Netherlands, Sweden, United Kingdom, Germany, Switzerland, and Italy) were evaluated for the risk of cancers[68]. The risk of colorectal cancer was not significantly higher (SIR 2.3; 95%CI: 0.9 to 6.2) in the overall cohort as well as in the subgroup patients who did not have any past history of cancer (SIR 1.4; 95%CI: 0.3 to 5.6). Although in the patients with a past history of cancer, the authors reported an increased risk of colorectal cancer (SIR 7.4; 95%CI: 1.9 to 29.7), the absolute number of cases with colorectal carcinoma was only 2. In the most recent study that combined the safety data from two large observational studies from the United States and Europe, the authors did not find any significant risk of GI malignancy with rGH treatment[69]. To summarize, although few studies showed increased risk of colorectal cancer, particularly in patients who were already in the high-risk group, the majority of the large cohort studies did not find any significant association between daily rGH therapy and increased risk of any GI cancer including colorectal adenocarcinoma[70].

Long-acting GH therapy (LAGH) has been approved by the United States Food and Drug Administration (lonapegsomatropin) for children with GH deficiency. However, there is theoretical concern regarding the possible risk of carcinogenic effect of increased IGF-1 level associated with LHGH therapy. Higher IGF-1 level is related to an increased risk of colon cancer in population-based studies [71]. However, none of the major human studies with LAGH therapy have reported any increased risk of colon cancer to date. In a recent meta-analysis, 11 studies with LHGH therapy also did not report any carcinogenic risk[72]. However, we need to remember that the follow-up duration of all published studies of LHGH therapy were short and inadequate to truly evaluate the long-term complications such as risk of cancer. Thus, future studies with long-term follow-up of children receiving LAGH therapy can only refute any possible carcinogenic effect of this molecule.

GI NEOPLASMS CAUSING ACROMEGALY

While we have so far discussed acromegaly leading to an increased risk of GI tumors, GI neoplasia causing acromegaly also can occur, although quite rare. Non-pituitary neoplasms causing GH excess is seen in less than 1% of all cases of acromegaly[73]. Most of these are due to ectopic GH-releasing hormone (GHRH)-secreting tumors. The most commonly reported GHRH-secreting tumors to date have been pancreatic neuroendocrine tumors and bronchial tumors[74-76]. In most of these cases, acromegaly

is clinically indistinguishable from that due to a pituitary adenoma. Pituitary imaging reveals pituitary hyperplasia and serum GHRH levels are elevated in most of the cases. Ectopic GH-secreting tumors causing acromegaly are even more rare. Of the reported cases, most have been by a pituitary adenoma located in the sphenoid sinus and very rarely by neoplasms at other sites such as non-Hodgkin's lymphoma[77,78].

CONCLUSION

Among the GI neoplasms, an intriguing link between colorectal neoplasms and acromegaly has been reported in most studies. However, the GI cancer-specific mortality rate was not found to be unequivocally elevated in acromegaly patients. Almost all guidelines advise an initial screening colonoscopy in acromegaly patients to facilitate the early diagnosis. Recommendations regarding the timing and frequency of repeat colonoscopy varies and needs individualization. The majority of large cohort studies did not report any significantly increased risk of gastrointestinal neoplasms in patients receiving rGH therapy.

FOOTNOTES

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Role of T-box transcription factor 3 in gastric cancers

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Abstract

The expression of T-box transcription factor 3 (TBX3) has been identified in various cancers, including gastric cancers. Its role in breast cancers and melanomas has been intensively studied, and its contribution to the progression of cancers through suppressing senescence and promoting epithelial-mesenchymal transition has been reported. Recent reports on the role of TBX3 in gastric cancers have implied its involvement in gastric carcinogenesis. Considering its pivotal role in the initiation and progression of cancers, TBX3 could be a promising therapeutic target for gastric cancers.

Key Words: Aging; Wnt; β -catenin; Transforming growth factor- β ; Stomach; Carcinogenesis

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Core Tip: Expression of T-box transcription factor 3 (TBX3) has been reported in a variety of cancers. Preceding reports have shown that TBX3 contributes to the progression of cancers by suppressing cellular senescence and promoting epithelial-mesenchymal transition. Recent reports on the role of TBX3 in gastric cancers have implied its involvement in aging-related gastric carcinogenesis.

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INTRODUCTION

The T-box gene family is involved in embryonic development[1] and is conserved among species[2]. Currently, seventeen transcription factors have been identified as members of the T-box gene family in mammals. The T-box gene family consists of five subfamilies, namely, T, Tbx1, Tbx2, Tbx6, and Tbr1 (Table 1).

T-box transcription factor 3 (TBX3), which belongs to the Tbx2 subfamily, was initially reported as the gene responsible for ulnar-mammary syndrome, an autosomal dominant human development disorder that affects limb, apocrine gland, tooth, hair, and genital development[3]. Studies with genetically engineered mice revealed that Tbx3 homozygous mutant mice were embryonic lethal and exhibited yolk sac defects, lack of mammary glands, and limb defects[4]. Subsequent studies have revealed the involvement of TBX3 in the development of numerous organs, including the heart[5], retina[6], ureter [7], and inner ear[8].

The TBX3 protein consists of 723 amino acids and is encoded by 2169 bp nucleotides in 7 exons. Differential splicing of the second intron leads to the addition of the 2a exon, resulting in the production of the TBX3+2a isoform. Both TBX3 and TBX3+2a are widely expressed in humans and mice, and alternative splicing of TBX3 was shown to be tissue- and species-specific[9]. TBX3 contains a DNA-binding T-domain[10], two repression domains, and an activation domain[11]. The protein is recruited to the T-box binding sites in the promoter regions of its downstream genes and acts both as a repressor and an activator. The functional similarity between TBX3 and its isoform is still controversial. Fan *et al* [9] reported that the TBX3+2a isoform lacked the ability to bind to the T-box binding site, and that while TBX3 immortalized mouse embryonic fibroblasts, the TBX3+2a isoform accelerated the senescence in those cells. On the other hand, Hoogaars *et al*[12] reported that both TBX3 and TBX3+2a were able to bind to the T-box binding site and inhibit cardiac chamber formation in mouse embryonic hearts. Another report from Zhao *et al*[13] showed that overexpression of either Tbx3 or Tbx3+2a induced the differentiation of mouse embryonic stem cells, but only Tbx3+2a was able to interact directly with Nanog. This discrepancy could be due to the difference in tissues and cells, and future studies are needed to elucidate this issue.

THE FUNCTION AND REGULATION OF TBX3

As expected from its broad expression, TBX3 has important functions. As mentioned earlier, it plays a crucial role in development. TBX3 binds to DNA through its T-domain, and functions as a repressor or an activator owing to its repression domains and an activation domain. Regarding cell cycle-related molecules, Tbx3 has been reported to repress p19^{ARF} (p14^{ARF} in humans) and inhibit cellular senescence [14,15]. This repression was either regulated through direct binding of TBX3 to the p14^{ARF} promoter[15] or through interactions of TBX3 with histone deacetylases (HDAC) 1, 2, 3, and 5[16]. Tbx3 has also been shown to suppress p53[17], while another preceding report demonstrated that it repressed p21^{CIP1/WAF} in a p53-independent manner[18]. In addition, Burgucu *et al*[19] reported that TBX3 suppressed phosphatase and tensin homolog by repressing its promoter activity, which led to augmented phosphatidylinositol-3-kinase activity. Collectively, these findings indicate that TBX3 possesses the ability to enhance cellular proliferation by regulating these molecules.

Several studies have reported that TBX3 suppresses apoptosis in addition to cellular senescence. Huang *et al*[20] showed that knocking down TBX3 in hypopharyngeal cancer cells increased annexin V-positive cells and the level of cleaved caspase 3. Ito *et al*[21] demonstrated that transfection of anti-sense Tbx3 into a rat bladder cancer cell line increased annexin V-positive cells, and the floating cells in the transfected culture exhibited DNA ladders on gel electrophoresis. These two previous studies reported that suppressing TBX3 led to increased apoptosis. On the other hand, Wensing and Campos[22] showed that overexpressing TBX3 and TBX3+2a reduced apoptosis in mesangial cells as assessed by caspase 3 activity. Carlson *et al*[17] also reported the anti-apoptotic function of TBX3 in overexpression experiments showing that transfection of TBX3 rescued primary mouse embryonic fibroblasts from Myc-induced apoptosis. Taken together, these preceding studies demonstrated that TBX3 possesses an anti-apoptotic function.

Another reported function of TBX3 is the repression of E-cadherin, which contributes to the promotion of epithelial-mesenchymal transition (EMT). Rodriguez *et al*[23] showed that TBX3 bound to the T-box binding site in the promoter of the *E-cadherin* gene and repressed E-cadherin expression, which resulted in enhanced invasiveness of melanomas. Dong *et al*[24] also reported that TBX3 repressed E-cadherin expression in hepatocellular carcinomas (HCC), but the repression occurred through the interaction of TBX3 with HDAC5. Peres *et al*[25] demonstrated that phosphorylation of TBX3 by AKT serine/threonine kinase 3 (AKT3) stabilized and promoted the nuclear translocation of TBX3, which was essential for E-cadherin repression. Although the effects were exerted through different mechanisms, these reports demonstrated that TBX3 negatively regulates E-cadherin expression, which can promote tumor invasion and metastasis.

Table 1 T-box gene family

T subfamily	Tbx1 subfamily	Tbx2 subfamily	Tbx6 subfamily	Tbr1 subfamily
T	Tbx1	Tbx2	Tbx6	Tbr1
Tbx19 (Tpit)	Tbx10	Tbx3	Mga	Tbr2 (Eomes)
	Tbx15	Tbx4		Tbx21 (Tbet)
	Tbx18	Tbx5		
	Tbx20			
	Tbx22			

For the regulation of TBX3, TBX3 has been regarded as one of the target genes of the Wnt/ β -catenin signaling pathway[7,26-28], and a preceding report showed that β -catenin directly bound to the Tcf binding site in the promoter region of *Tbx3* and induced *Tbx3* expression[29]. However, the Wnt/ β -catenin signaling pathway is not the only signaling pathway that regulates *Tbx3* expression. Transforming growth factor- β (TGF- β) is another signaling molecule that has been shown to induce *Tbx3*. Li *et al*[30] showed that Smad3 and Smad4, downstream signaling molecules of TGF- β , bound to the Smad-binding element in the *TBX3* promoter together with JunB and enhanced *TBX3* promoter activity. Lee *et al*[31] also reported that TBX3 was upregulated by TGF- β , although they demonstrated that this induction was dependent on the MAPKK-like protein kinase TOPK. Notch signaling has been shown to facilitate the nuclear translocation of Smad3 and activate TGF- β signaling[32,33], and considering that Notch signaling activates *Tbx5*[34], another member of the Tbx2 subfamily, it is possible that Notch signaling also regulates *Tbx3* expression, but further studies are needed to clarify whether Notch signaling regulates TBX3 expression (Figure 1).

TBX3 IN BREAST CANCERS AND MELANOMAS

Overexpression of TBX3 has been reported in various cancers[35]. Among them, breast cancers and melanomas are the cancers in which the role of TBX3 has been intensively studied.

Sequencing of 100 primary breast cancers identified driver mutations in several genes, including *TBX3*[36], and another comprehensive study of 817 breast tumors identified that mutations in *TBX3* were enriched in invasive breast cancers[37]. In addition, genomic sequencing of 1918 breast cancers also indicated that alterations in *TBX3* were enriched in breast cancers[38]. These studies demonstrated that TBX3 is one of the key players in breast cancers. Recently, Kostecka *et al*[39] reported that sequencing of cancer-associated genes, including *TBX3*, in normal mammary glands of 52 patients with reportedly sporadic breast cancer revealed that subclonal somatic pathogenic variants of these genes were identified at considerable allelic frequencies. This suggests that TBX3 plays an important role in the initiation of breast cancers.

Functionally, TBX3 has been shown to promote the progression of breast cancers by suppressing cellular senescence and enhancing EMT, as described earlier in this review. However, although overexpression of TBX3 alone accelerated mammary epithelial cell proliferation and led to mammary gland hyperplasia, it did not lead to tumor development[40], which implies that overexpression of TBX3 alone is inadequate to initiate breast cancers.

Preceding studies have also reported the overexpression of TBX3 in melanomas[23,25,41], and interestingly, the constitutively active *B-RAF* mutation observed in melanomas was reported to induce TBX3[42]. Recently, a comprehensive study of 189 cohorts and 178 individual patients identified TBX3 as a marker of poorly differentiated melanomas[43]. Mechanistically, TBX3 was determined to promote tumor progression through inhibition of cellular senescence and promotion of EMT, similar to its role in breast cancers. However, in contrast to its role in breast cancers, the overexpression of TBX3 alone was sufficient to promote the formation and invasion of melanomas[44].

Taken together, the preceding studies suggested that TBX3 promotes tumor progression and invasion by suppressing senescence and enhancing EMT, but whether TBX3 can initiate cancers seems to depend on the type of cancer.

TBX3 IN COLORECTAL CANCERS, PANCREATIC CANCERS, AND LIVER CANCERS

The involvement of TBX3 has also been reported in colorectal cancers. A genome-wide meta-analysis revealed the association of polymorphisms in *TBX3* with increased colorectal tumor risk[45]. Shan *et al* [46] reported that aberrant TBX3 expression was associated with a large tumor size, poor differentiation,

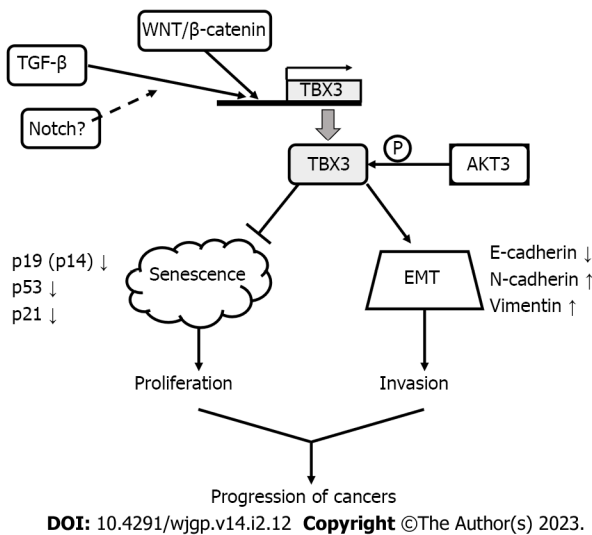


Figure 1 The role of T-box transcription factor 3 in cancers. A schema describing the regulation and function of T-box transcription factor 3 in cancers. P: Phosphorylation; EMT: Epithelial-mesenchymal transition; TGF-β: Transforming growth factor-β; TBX3: T-box transcription factor 3.

invasion, lymph node metastasis, and advanced TNM stage in colorectal cancers, resulting in poor prognosis. They also showed through multivariate analysis that TBX3 can independently predict the outcome of colorectal cancer patients.

Similar to the findings in colorectal cancers, augmented TBX3 expression was associated with poor prognosis in pancreatic cancer patients and was reported to be an independent prognostic factor for overall survival[47]. Regarding the mechanism of TBX3, Perkhofer *et al*[48] demonstrated that TBX3 enhanced migration, invasion, and angiogenesis in pancreatic cancers through *in vitro* and *in vivo* studies.

TBX3 has also been reported to be associated with histological grade, tumor size, metastasis, and Ki-67 expression in HCC[49] and the expression of TBX3 in HCC was found to be induced by Wnt/β-catenin signaling[26,29,50]. Interestingly, Tbx3 in the hepatic microenvironment has been reported to play a crucial role in determining the fate of transformed hepatic cells and whether they develop HCC or intrahepatic cholangiocarcinoma[51].

Collectively, these studies demonstrated that TBX3 plays a major role in these cancers.

TBX3 IN GASTRIC CANCERS

Concerning gastric cancers, Miao *et al*[52] reported that TBX3 was overexpressed in 46 of 98 primary gastric cancer tissues, and its overexpression correlated with advanced TNM stage and with a higher relapse incidence. *In vitro* studies demonstrated that overexpression of TBX3 augmented cellular proliferation, whereas knockdown of TBX3 suppressed proliferation in gastric cancer cell lines. Regarding the mechanism involved in TBX3-induced accelerated proliferation, they showed that TBX3 overexpression led to a reduction in the percentage of cells in G1 phase and an increase in the percentage of cells in S and G2 phases in addition to augmented c-Myc and cyclin D1 expression, suggesting that TBX3 facilitated cell cycle progression. The *in vitro* studies also indicated that TBX3 downregulated E-cadherin and induced N-cadherin and vimentin expression, which suggested the enhancement of EMT. This enhancement of proliferation and EMT could be the reason why the expression of TBX3 is associated with advanced tumor stage in gastric cancers, similar to its correlation with poor prognosis in colorectal cancer patients[46].

Takeuchi *et al*[53] recently reported the essential role of Tbx3 in aging-related gastric carcinogenesis. Analysis of gastric organoids established from young and aged mice revealed that cellular proliferation was enhanced in aged gastric organoids due to Tbx3-induced repression of cellular senescence. Aged gastric organoids exhibited suppressed expression of Dickkopf3 (Dkk3), a Wnt antagonist, due to methylation of the *Dkk3* gene, and consequently, the enhanced Wnt/β-catenin signaling induced Tbx3 expression. Epigenetic alterations, such as the methylation of the *Dkk3* gene, are considered as one of the hallmarks of aging[54]. The stochastic process that involves alterations of the methylation state over time is referred to as epigenetic drift and is considered to track biological tissue aging[55]. Indeed, Takeuchi *et al*[53] showed that DKK3 expression in human gastric tissues decreased as the patient aged, whereas TBX3 expression in human gastric tissues exhibited a positive correlation with patient age. Furthermore, they showed that gastric cancer tissues exhibited lower DKK3 expression and higher TBX3 expression than normal oxyntic glands, suggesting the central role of TBX3 in aging-related gastric

carcinogenesis.

Another study of gastric precancerous lesions in 449 patients identified *TBX3*, along with *CDX2* and *MYC*, as one of the top 7 core genes that contributed to the progression from low-grade intraepithelial neoplasia to high-grade intraepithelial neoplasia[56], a finding that emphasizes the involvement of *TBX3* in the early stage of gastric carcinogenesis.

Taken together, these studies imply that *TBX3* plays a pivotal role in aging-related carcinogenesis and the progression of gastric cancers. Further studies are awaited to confirm the role of *TBX3* in aging-related gastric carcinogenesis.

TBX3 AS A THERAPEUTIC TARGET

Since *TBX3* is expressed in various cancers and possesses the ability to promote the progression of these tumors, it has been considered a therapeutic target in these cancers[57]. As *TBX3* has been shown to promote cancer progression, its suppression will be required for therapies. Several microRNAs (miR) have been reported to inhibit *TBX3*. In adipocytes, miR-93 has been shown to inhibit *TBX3* and negatively control adipogenesis[58]. On the other hand, miR-137 was reported to inhibit *TBX3* in breast cancers[59] and melanomas[60]. In pancreatic cancers, members of the miR-17-92 cluster have been shown to inhibit *TBX3* together with p21 and p57[61]. Furthermore, miR-183 was found to suppress *TBX3* and enhanced sensitivity to chemotherapy in laryngeal cancers[62]. These miRNAs could be considered candidates for the treatment of *TBX3*-expressing cancers.

Concerning chemical reagents, an integrated computational approach indicated that two alkaloids, Jervine and Diflomotecan, can form stable complexes with *TBX3* and suggested them as new effective drugs against breast cancers[63]. In another study, an aqueous extract of *Fructus ligustri lucidi*, a common Chinese herbal medicine, was reported to suppress *TBX3* and enhance sensitivity to doxorubicin in colon cancer cells[64].

Recently, Willmer *et al*[65] reported that the multifunctional phosphoprotein nucleolin is required for *TBX3* to function and that the nucleolin-targeting aptamer AS1411 exhibited an anticancer effect against sarcomas. These reagents could contribute to anticancer therapy against *TBX3*-overexpressing cancers.

In addition to its role as a therapeutic target, *TBX3* can also contribute to treatment by aiding in the selection of medication for chemotherapy. Freeman *et al*[43] proposed using *TBX3* to predict the outcomes of immune checkpoint inhibitors against melanomas. They showed that patient stratification into risk groups regarding *TBX3* and *MAP4K1* expression was associated with overall survival; hence, evaluating the expression of these genes could enable individualized treatment for each patient. Similar findings may be found in other *TBX3*-expressing cancers, and further studies are warranted.

CONCLUSION

In this review, we discussed the role of *TBX3* in cancers. *TBX3* is expressed in various cancers and contributes to their progression mainly through the repression of senescence and the promotion of EMT. Given its crucial role in tumor progression, *TBX3* could be a promising therapeutic target in malignant tumors, including gastric cancers.

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Case Control Study

Polymorphism of genes encoding drug-metabolizing and inflammation-related enzymes for susceptibility to cholangiocarcinoma in Thailand

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Abstract

BACKGROUND

Cholangiocarcinoma (CCA) is an intractable cancer, and its incidence in north-

eastern Thailand is the highest worldwide. Infection with the liver fluke *Opisthorchis viverrini* (OV) has been associated with CCA risk. However, animal experiments have suggested that OV alone does not induce CCA, but its combination with a chemical carcinogen like nitrosamine can cause experimentally induced CCA in hamsters. Therefore, in humans, other environmental and genetic factors may also be involved.

AIM

To examine relations between risk for CCA and genetic polymorphisms in carcinogen-metabolizing and inflammation-related genes.

METHODS

This hospital-based case-control study enrolled 95 case-control pairs matched by age (± 5 years) and sex. We examined relations between risk for CCA and genetic polymorphisms in carcinogen-metabolizing and inflammation-related genes, serum anti-OV, alcohol consumption, and smoking. Polymorphisms of CYP2E1, IL-6 (-174 and -634), IL-10 (-819), and NF- κ B (-94) and their co-occurrence with polymorphisms in the drug-metabolizing enzyme gene GSTT1 or GSTM1 were also analyzed.

RESULTS

Although CCA risk was not significantly associated with any single polymorphism, persons with the GSTT1 wild-type and CYP2E1 c1/c2 + c2/c2 genotype had an increased risk (OR = 3.33, 95%CI: 1.23-9.00) as compared with persons having the GSTT1 wild-type and CYP2E1 c1/c1 wild genotype. The presence of anti-OV in serum was associated with a 7- to 11-fold increased risk, and smoking level was related to an OR of 1.5-1.8 in multivariable analyses adjusted for each of the seven genetic polymorphisms.

CONCLUSION

In addition to infection with OV, gene-gene interactions may be considered as one of the risk factors for CCA development.

Key Words: *Opisthorchis*; Glutathione transferase; Cytochrome P-450 CYP2E1; Case-control study

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Core Tip: Cholangiocarcinoma (CCA) is an intractable cancer, and its prevalence in northeastern Thailand is the highest worldwide. An inflammatory condition produced by infection with the liver fluke *Opisthorchis viverrini* (OV) has been associated with CCA risk, but the susceptibility of individuals has not been fully examined. Our study revealed that persons with the GSTT1 wild-type and CYP2E1 c1/c2 + c2/c2 genotype had an increased risk for developing CCA (OR = 3.33, 95%CI: 1.23-9.00). Therefore, both gene-gene interactions and OV infection should be considered as risk factors for cholangiocarcinogenesis.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a cancer of the hepatobiliary tract, and its incidence is extremely high in northeastern Thailand[1]. This is related to the lifestyle of the people of this area who often consume raw fish, which carries the risk of ingesting fish-borne parasites[2]. Infection with the liver fluke, *Opisthorchis viverrini* (OV), is a known CCA risk factor for inhabitants of northeastern Thailand[3-5], where approximately one-third of the population has been infected with OV[6,7]. Although infection with OV has been listed as a carcinogen to humans by The International Agency for Research on Cancer (IARC)[8], infection with OV alone is not sufficient for CCA development. Indeed, experiments with hamsters have suggested that parasitic infection alone is not sufficient to develop CCA. In fact, co-administration of a chemical carcinogen such as *N*-nitrosodimethylamine is necessary to induce CCA in hamsters[9]. In addition, genetic background related to the activation or detoxification of chemical

carcinogens is reported to be involved in CCA risk[10]. Also, elevated plasma IL-6 was associated with increased risk of CCA in patients infected with OV[11]. Thus, maintenance of chronic infection, exposure to a chemical carcinogen(s), and genetic background may explain CCA risk in northeastern Thailand. We previously reported that infection with OV and genetic polymorphism of a drug-metabolizing enzyme gene, namely *GSTM1*, is related to CCA risk[5] and that the combined effect of polymorphisms of the genes *8-oxoguanine glycosylase 1* and *GSTM1* is also relevant CCA risk[12] in northeastern Thailand. Here, we report our analysis of the association between polymorphisms of inflammation-related genes (*IL-6*, *IL-10* and *NF-kB*), and *CYP2E1*, *GSTT1* and *GSTM1* with risk of developing CCA. We also analyzed potential interactions among genetic polymorphisms of these genes.

MATERIALS AND METHODS

This work was conducted after receiving the approval from the ethics committees of the Nagahama Institute of Bio-Science and Technology, Shiga, Japan, and the National Cancer Institute, Bangkok, Thailand.

Study subjects

All cases with CCA were identified between 1999 and 2005 upon a visit to the Ubon Ratchathani Cancer Centre in the northeastern province of Thailand, one of the cancer centers administered by the National Cancer Institute of Thailand. Diagnosis was based on abdominal ultrasonography by a single radiologist at Ubon Ratchathani Cancer Centre with serological supportive evidence including an elevated carbohydrate antigen 19-9 level (≥ 40 μ /mL). Each case was matched by sex and age (within 5 years) with each control subject who lived in the same Ubon Ratchathani area and visited Ubon Ratchathani Cancer Centre for health check-up. All control subjects were without any clinical, ultrasonographical, or serological abnormalities. Finally, the 95 case-control pairs having data for an antibody against infection with OV were employed although the number of subjects for each of the analyses involving genetic polymorphisms was not equal because the amount of blood samples was limited. Still, there was a narrow range (91-95) for the number of case-control pairs for each of the genetic polymorphisms examined (Table 1).

OV infection

Infection with OV was determined with an enzyme-linked immunosorbent assay using an antibody ("anti-OV") raised against an OV antigen[13].

DNA extraction

Blood samples were frozen and stored at -80°C. DNA was extracted from 2 mL blood with the QIAGEN DNA Blood Midi kit[14].

Analysis of *GSTM1* and *GSTT1* polymorphisms

Polymorphisms in each of *GSTM1* and *GSTT1* were determined with polymerase chain reaction (PCR) [5] using the following primers: *GSTM1*, 5'-GAA CTC CCT GAA AAG CTA AAG C-3' and 5'-GTT GGG CTC AAA TAT ACG GTG G-3'; *GSTT1*, 5'-TTC CTT ACT GGT CCT CAC ATC TC-3' and 5'-TCA CCG GAT CAT GGC CAG CA-3'; *CYP1A1* (internal control), 5'-GAA CTG CCA CTT CAG CTG TCT-3' and 5'-CAG CTG CAT TTG GAA GTG CTC-3'. Each 20- μ L reaction contained 0.2 μ M of each primer, 200 μ M deoxyribonucleoside triphosphates (dNTPs), 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.5), 1.5 mmol/L $MgCl_2$, approximately 6 ng DNA, and *Taq* DNA polymerase (Ampli Taq Gold; Cetus PerkinElmer, Norwalk, CT). The PCR cycling parameters were 3 min at 95°C (initial denaturation) followed by 45 cycles of 1 min at 95°C, 30 sec at 59°C, and 1 min at 72°C, with a final 10-min extension step. The PCR products (299 bp for *GSTM1*, 507 bp for *GSTT1*) were separated on a 2% agarose gel.

Analysis of *CYP2E1* polymorphisms

The 5'-flanking *RsaI* site polymorphism of *CYP2E1* was detected by PCR combined with restriction-fragment-length polymorphism analysis (PCR-RFLP). The forward and reverse primers were 5'-CCA GTC GAG TCT ACA TTG TCA-3' and 5'-TTC ATT CTG TCT TCT AAC TGG-3', respectively. Each 20- μ L reaction contained 0.2 μ M of each primer, 200 μ M dNTPs, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.5), 1.5 mmol/L $MgCl_2$, approximately 6 ng DNA, and *Taq* DNA polymerase. The PCR cycling parameters were 12 min at 95°C (initial denaturation) followed by 40 cycles of 1 min at 95°C, 30 sec at 60°C, and 1 min at 72°C, with a final 10-min extension step at 72°C. A 10- μ L aliquot of the PCR products was digested with 0.2 U of the restriction enzyme *RsaI* (GTAU) (TOYOBO Co., Ltd., Japan) for 16 h at 37°C. The resulting fragments were separated by 3% agarose gel electrophoresis and stained with ethidium bromide. The expected PCR product was 410 bp. Upon digestion with *RsaI*, the c1/c1 wild-type homozygotes had fragments of 360 and 50 bp, the c1/c2 heterozygotes had fragments of 410, 360 and 50 bp, and the c2/c2 variant homozygotes had a fragment of 410 bp[15].

Table 1 Number of subjects with non-missing and with missing values for serum anti-*Opisthorchis viverrini*, alcohol consumption, and smoking dependent on each matched-pair analysis

Principle independent variable	Number of matched pairs	Anti-OV		Alcohol consumption		Smoking	
		Missing	Non-missing	Missing	Non-missing	Missing	Non-missing
Anti-OV	95	Not applicable		3	187	2	188
GSTM1	95	10	180	2	188	2	188
GSTT1	95	10	180	2	188	2	188
CYP2E1	93	9	177	2	184	2	184
IL6, -634 G/C	91	10	172	2	180	2	180
IL6, -175 G/C	92	10	174	2	182	2	182
IL10, -819 T/C	91	10	172	2	180	2	180
NF-κB, -94 ins/del ATTG	92	10	174	2	182	2	182

OV: *Opisthorchis viverrini*.

Analysis of IL-6 (rs1800795) polymorphisms

Polymorphism -174 (G/C) (rs1800795) of *IL-6* was detected by PCR-RFLP with forward primer 5'-ATG CCA AGT GCT GAG TCA CTA-3' and reverse primer 5'-TCG AGG GCA GAA TGA GCC TC-3'. Each 20-μl reaction contained 0.2 μM of each primer, 200 μM dNTPs, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl₂, approximately 6 ng DNA, and *Taq* DNA polymerase. The PCR cycling parameters were 10 min at 95°C (initial denaturation) followed by 40 cycles of 30 sec at 95°C, 30 sec at 54°C, and 40 sec at 72°C, with a final 10-min extension step at 72°C. A 10-μl aliquot of the PCR products was digested with 0.2 U of the restriction enzyme *Nla*III (CATG↓) (New England Biolabs, Japan) at 37°C for 16 h and separated by PAGE (10% polyacrylamide gel). The expected PCR product was 308 bp. Upon digestion with *Nla*III, the G/G wild-type homozygotes had fragments of 233 and 75 bp, the G/C heterozygotes had fragments of 233, 121, 112 and 75 bp, and the C/C variant homozygotes had fragments of 121, 112 and 75 bp[16].

Analysis of IL-6 (rs1800796) polymorphisms

Polymorphism -634 G/C (rs1800796) of *IL-6* was analyzed by PCR with confronting two-pair primers [17]. The four primers used were *IL-6-634 F1* 5'-CCT CTA AGT TGG GCT GAA GCA GG-3' and *IL-6-634 R1* 5'-GTT CTG GCT CTC CCT GTG AGG-3' for amplifying the variant type, and *IL-6-634 F2* 5'-CCA GGC AGT TCT ACA ACA GCC G-3' and *IL-6-634 R2* 5'-TGA GTT TCC TCT GAC TCC ATC GC-3' for amplifying wild type. Each 25-μl reaction contained 0.2 μM of each primer, 200 μM dNTPs, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2 mmol/L MgCl₂, approximately 6 ng DNA, and *Taq* DNA polymerase. The PCR cycling parameters were 10 min at 95°C (initial denaturation) followed by 36 cycles of 1 min at 95°C, 1 min at 65°C, and 1 min at 72°C, with a final 5-min extension step at 72°C. The PCR products were separated by PAGE. The G/G wild-type homozygotes had fragments of 157 and 240 bp, G/C heterozygotes had fragments of 125, 157, 240 and 75 bp, and the C/C variant homozygotes had fragments of 125 and 240 bp.

Analysis of IL-10 (rs1800871) polymorphisms

Polymorphism -819 T/C (rs1800871) of *IL-10* was analyzed by PCR-RFLP. The forward and reverse primers were *IL-10-819 F* 5'-TCA TTC TAT GTG CTG GAG ATG G-3' and *IL-10-819 R* 5'-TGG GGG AAG TGG GTA AGA GT-3', respectively. Each 20-μl reaction contained 0.2 μM of each primer, 200 μM dNTPs, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2 mmol/L MgCl₂, approximately 6 ng DNA, and *Taq* DNA polymerase. The PCR cycling parameters were 5 min at 95°C (initial denaturation) followed by 40 cycles of 30 sec at 95°C, 45 sec at 59°C, and 1 min at 72°C, with a final 10-min extension step at 72°C. A 10-μl aliquot of the PCR products was digested with 0.03 U of the restriction enzyme *Mae* III (↓GTNAC) (Roche Diagnostics GmbH Germany) for 16 h at 55°C and separated by PAGE. The expected PCR product was 209 bp. Following digestion with *Mae*III, the T/T wild-type homozygotes had a fragment of 209 bp, the T/C heterozygotes had fragments of 209, 125 and 84 bp, and the C/C variant homozygotes had fragments of 209 and 84 bp[18].

Analysis of NF-κB polymorphisms

Polymorphism -94 ins/del ATTG of the *NF-κB* promoter was determined by PCR-RFLP. The forward and reverse primers were 5'-TTT AAT CTG TGA AGA GAT GTG AAT-3' and 5'-CTA GCA GGG CGC TCC CGA AT-3', respectively. Each 20-μl reaction contained 0.2 μM of each primer, 200 μM dNTPs, 50

Table 2 Relationship between serum anti-*Opisthorchis viverrini* and cholangiocarcinoma risk, Ubon Ratchathani, Thailand

Number of case-control pairs according to serum anti-OV status: positive (+) vs negative (-)				Crude OR	95%CI		P value	Adjusted ¹ OR	95%CI		P value	Adjusted ² OR	95%CI		P value
Case (+) / Control (+)	Case (+) / Control (-)	Case (-) / Control (+)	Case (-) / Control (-)		LL	UL			LL	UL			LL	UL	
67	23	2	3	11.50	2.71	48.78	< 0.001	8.96	2.06	38.99	0.001	9.40	2.16	40.85	0.003

¹Adjusted for alcohol consumption and smoking.²Adjusted for alcohol consumption and smoking. Missing values for these variables were imputed.OV: *Opisthorchis viverrini*; CCA: Cholangiocarcinoma; LL: Lower limit; UL: Upper limit.

mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl₂, approximately 6 ng DNA, and *Taq* DNA polymerase. The PCR cycling parameters were 10 min at 95°C (initial denaturation) followed by 40 cycles of 30 sec at 95°C, 1 min at 56°C, and 1 min at 72°C, with a final 10-min extension at 72°C. A 10-μl aliquot of the PCR products was digested with 2 U of the restriction enzyme *Pfl*MI (New England Biolabs Inc., Japan) at 37°C for 16 h and separated by PAGE. The expected PCR product was 254 bp. Upon digestion with *Pfl*MI, the del/del ATTG wild-type homozygotes had a fragment of 254 bp, the ins/del heterozygotes had fragments of 254 and 206 bp, and the ins/ins variant homozygotes had a fragment of 206 bp[19].

Data collection for lifestyle-related factors

Smoking and alcohol consumption status were ascertained alongside dietary habits at the hospital by local health personnel using a structured questionnaire used in previous studies[5,12]. Smoking status was classified into four categories: Never, occasional, former, and current. A current smoker was defined as smoking at least 1 cigarette *per* day, whereas the former smoker was defined as having stopped smoking regularly at least 1 year prior to our study. Alcohol consumption status was classified the same as for smoking status into four categories: Never, occasional, former, and current. “Current” drinker was defined as drinking more than once a week, whereas “former” drinker was defined as having stopped regular drinking at least 1 year prior to the study.

Statistical analysis

CCA risk attributable to infection with OV was defined as positivity for anti-OV, and the potential contributions of genetic polymorphisms of genes encoding inflammation-related enzymes were examined by calculating an OR. Each OR was calculated using a conditional logistic regression model, keeping matched case-control pairs. Interactions between each of the genetic polymorphisms of the inflammation-related genes and each of the metabolic enzyme genes were first examined using a log-likelihood ratio test. The tests compared the main effect with no interaction terms with a full model that included the interaction term for the variables concerned. When the p value for the log-likelihood ratio statistic was < 0.1, we calculated the OR owing to the co-occurrence of two genetic polymorphisms. Three subjects lacked data for alcohol consumption, and two lacked data for smoking status (Table 1). Most of the multivariable statistical analyses were performed with imputation for the missing data for these five subjects and produced comparable results (Tables 2-4). All statistical analyses were performed

Table 3 Relationship between serum anti-*Opisthorchis viverrini*, alcohol consumption, and smoking and cholangiocarcinoma risk, Ubon Ratchathani, Thailand, shown by analysis for each of the genetic polymorphisms

Independent variable	Gene adjusted for	Adjusted ¹ OR	95%CI		P value	Adjusted ² OR	95%CI		P value
			LL	UL			LL	UL	
Positive serum anti-OV	<i>GSTM1</i>	9.16	2.00	41.90	0.004	7.12	1.76	28.81	0.006
	<i>GSTT1</i>	8.16	1.85	35.96	0.006	7.33	1.74	30.93	0.007
	<i>CYP2E1</i>	7.75	1.73	34.69	0.007	4.90	1.41	17.05	0.013
	<i>IL-6</i> , -174 G/C	8.07	1.80	36.07	0.006	5.39	1.51	19.29	0.010
	<i>IL-6</i> , -634 G/C	7.21	1.57	32.98	0.011	6.13	1.54	24.32	0.010
	<i>IL-10</i> , -819 T/C	9.04	1.97	41.39	0.005	6.03	1.65	22.04	0.007
	<i>NF-κB</i>	11.81	2.31	60.44	0.003	8.14	1.90	34.90	0.005
Alcohol drinking	<i>GSTM1</i>	1.06	0.70	1.60	0.779	1.12	0.76	1.67	0.560
	<i>GSTT1</i>	1.05	0.70	1.58	0.817	1.10	0.74	1.63	0.643
	<i>CYP2E1</i>	1.05	0.70	1.58	0.801	1.10	0.75	1.63	0.614
	<i>IL-6</i> , -174 G/C	1.03	0.68	1.56	0.907	1.09	0.73	1.62	0.674
	<i>IL-6</i> , -634 G/C	1.00	0.65	1.52	0.989	1.06	0.70	1.59	0.784
	<i>IL-10</i> , -819 T/C	0.98	0.63	1.53	0.933	1.06	0.70	1.61	0.785
	<i>NF-κB</i>	0.99	0.63	1.54	0.956	1.05	0.68	1.60	0.836
Smoking	<i>GSTM1</i>	1.56	1.01	2.40	0.044	1.49	0.99	2.23	0.053
	<i>GSTT1</i>	1.56	1.01	2.42	0.044	1.51	1.00	2.27	0.049
	<i>CYP2E1</i>	1.55	1.00	2.41	0.052	1.46	0.98	2.18	0.060
	<i>IL-6</i> , -174 G/C	1.73	1.05	2.85	0.030	1.62	1.04	2.54	0.035
	<i>IL-6</i> , -634 G/C	1.79	1.10	2.91	0.018	1.73	1.08	2.76	0.022
	<i>IL-10</i> , -819 T/C	1.80	1.09	2.98	0.022	1.65	1.05	2.59	0.030
	<i>NF-κB</i>	1.78	1.08	2.94	0.023	1.80	1.11	2.91	0.016

¹Adjusted for alcohol consumption and smoking.²Adjusted for alcohol consumption and smoking. Missing values for these variables were imputed.OV: *Opisthorchis viverrini*; CCA: Cholangiocarcinoma; LL: Lower limit; UL: Upper limit.

with the statistical package STATA 16.1 (College Station, TX, USA), and statistical significance was defined as $P < 0.05$ unless indicated otherwise.

Although the CCA-related risk of infection with OV among the current study subjects was reported in the multivariable analyses that included the interaction terms between the *GSTM1* and *hOGG1* polymorphisms in our previous study[12], a crude or adjusted OR for each polymorphism has not been reported. Data for CCA risk owing to polymorphism of *GSTM1* or *GSTT1* alone also has not been reported[12]. Findings for those factors are, thus, reported in the present study.

RESULTS

Concerning environmental factors, we confirmed the strong association between anti-OV-positive individuals and CCA risk adjusted for smoking status and alcohol consumption (Table 2). The OR values (95%CI) for a one-category change in smoking category (never, occasional, former, current) were approximately 1.5 regardless of the particular genetic polymorphism(s) examined or whether imputed data were included (Table 3). Alcohol consumption was not materially related to CCA risk (Table 3).

There was no significant association between polymorphisms for any of the inflammation-related genes or drug-metabolizing genes and CCA risk. When an inflammation-related gene (*IL-6*, *IL-10*, *NF-κB*) wild-type genotype was used as the reference, CCA risk was not significantly associated with any homozygous or heterozygous genotype (Table 4). Likewise, when the *GSTT1* wild-type genotype was used as the reference, CCA risk was not significantly associated with the null genotype. Similarly, when

Table 4 Relationship between polymorphisms of drug-metabolizing and inflammation-related enzyme genes and cholangiocarcinoma risk, Ubon Ratchathani, Thailand

Gene	Number of pairs according to gene polymorphism of case and control					Polymorphism	Crude OR	95%CI		P value	Adjusted ¹ OR	95%CI		P value	Adjusted ² OR	95%CI		P value
								LL	UL			LL	UL			LL	UL	
GSTM1																		
			Case															
			Wild	Null														
	Control	Wild	8	18		Wild	1.00	Reference			1.00	Reference			1.00	Reference		
		Null	18	51		Null	1.00	0.52	1.92	1.000	0.67	0.29	1.56	0.357	0.81	0.37	1.75	0.594
GSTT1																		
			Case															
			Wild	Null														
	Control	Wild	44	23		Wild	1.00	Reference			1.00	Reference			1.00	Reference		
		Null	21	7		Null	1.10	0.61	1.98	0.763	0.94	0.44	1.98	0.865	1.02	0.52	2.02	0.957
CYP2E1																		
			Case															
			c1/c1	c1/c2	c2/c2													
	Control	c1/c1	56	14	4	c1/c1	1.00	Reference			1.00	Reference			1.00	Reference		
		c1/c2	12	4	2	c1/c2	1.11	0.52	2.38	0.782	1.02	0.39	2.70	0.966	1.14	0.47	2.77	0.773
		c2/c2	1	0	0	c2/c2	6.19	0.74	52.08	0.093	2.02	0.20	20.67	0.552	4.17	0.45	38.24	0.207
							c1/c2 or c2/c2	1.38	0.68	2.83	0.371	1.14	0.47	2.77	0.767	1.35	0.58	3.11
IL-6																		
			Case			-174												
			G/G	G/C	C/C													
	Control	G/G	90	0	0	G/G	1.00	Reference			1.00	Reference			1.00	Reference		
		G/C	1	0	0	G/C	0.00	-	-	-	0.003	0.00	-	0.995	0.0037	0.00	-	0.999
		C/C	0	0	0	C/C	-	-	-	-	-	-	-	-	-	-	-	-
IL-6																		
						-634												

		Case																
		G/G	G/C	C/C														
IL-10	Control	G/G	0	4	5	G/G	1.00	Reference			1.00	Reference			1.00	Reference		
		G/C	2	13	12	G/C	1.93	0.63	5.93	0.252	1.90	0.49	7.30	0.351	1.69	0.46	6.25	0.432
		C/C	4	18	34	C/C	1.28	0.44	3.73	0.654	1.38	0.40	4.71	0.607	1.45	0.43	4.87	0.551
						G/C or C/C	1.50	0.53	4.21	0.442	1.53	0.46	5.05	0.488	1.52	0.46	4.98	0.489
						-819 T/C												
		Case																
		T/T	T/C	C/C														
NF- κ B	Control	T/T	23	15	6	T/T	1.00	Reference			1.00	Reference			1.00	Reference		
		T/C	15	17	4	T/C	0.81	0.41	1.60	0.537	0.66	0.27	1.63	0.371	0.75	0.34	1.70	0.497
		C/C	8	0	3	C/C	1.19	0.46	3.06	0.715	1.59	0.43	5.93	0.487	1.63	0.51	5.17	0.405
						T/C or C/C	0.91	0.51	1.65	0.763	0.85	0.39	1.87	0.686	0.95	0.47	1.93	0.891
						-94 ins/del ATTG												
		Case																
		del/del	ins/del	ins/ins														
	Control	del/del	3	5	3	del/del	1.00	Reference			1.00	Reference			1.00	Reference		
		ins/del	4	16	13	ins/del	0.83	0.32	2.17	0.711	1.11	0.27	4.61	0.884	0.79	0.24	2.67	0.708
		ins/ins	9	20	19	ins/ins	0.48	0.19	1.23	0.128	0.32	0.08	1.20	0.090	0.33	0.10	1.03	0.057
						ins/del or ins/ins	0.91	0.51	1.65	0.763	0.49	0.14	1.69	0.258	0.47	0.16	1.38	0.167

¹Adjusted for alcohol consumption and smoking.

²Adjusted for alcohol consumption and smoking. Missing values for these variables were imputed.

CCA: Cholangiocarcinoma; LL: Lower limit; UL: Upper limit.

the *GSTM1* wild-type genotype was used as the reference, CCA risk was not significantly associated with the null genotype. When the *CYP2E1* c1/c1 wild-type genotype was used as the reference, CCA risk was not significantly associated with c1/c2, c2/c2, or the combination of the c1/c2 + c2/c2 genotypes (Table 4).

Gene-gene interactions between *GSTT1* and *CYP2E1*

Interaction of the *CYP2E1* polymorphism and the *GSTT1* polymorphisms was suggested by a p value of

Table 5 Risk of cholangiocarcinoma due to the combination of polymorphisms of GSTT1 and CYP2E1: Matched case-control study, Ubon Ratchathani, Thailand

CYP2E1	GSTT1 wild		GSTT1 null		CYP2E1	GSTT1 wild			GSTT1 null					
	Number		Number			Odds ratio [†]	95%CI		P value	Odds ratio [†]	95%CI		P value	P for interaction
	Case	Control	Case	Control			LL	UL			LL	UL		
c1/c1	49	59	25	18	c1/c1	1.00	Reference		2.04	0.95	4.39	0.07	< 0.01	
c1/c2 + c2/c2	24	9	5	11	c1/c2 + c2/c2	3.33	1.23	9.00	0.02	0.54	0.14	2.13	0.38	

¹Based on the conditional logistic regression model.

CCA: Cholangiocarcinoma; CI: Confidence interval; LL: Lower limit; UL: Upper limit.

0.003, whereas other interactions between drug-metabolizing enzyme genes (*GSTM1* or *GSTT1*) and other genetic polymorphisms were not substantiated ($P > 0.10$). Persons with the *GSTT1* wild-type plus *CYP2E1* c1/c2 + c2/c2 genotype had an increased risk for CCA (OR = 3.33, 95%CI: 1.23-9.00) as compared with persons having the *GSTT1* wild-type plus *CYP2E1* c1/c1 wild-type genotype that was used as the reference (Table 5).

DISCUSSION

Early diagnosis of CCA is difficult, and most patients die within a year after diagnosis[20]. Identification of risk factors and means of preventing cholangiocarcinogenesis is thus highly desirable. We confirmed that positivity for anti-OV constitutes a significant risk factor for CCA, as reported previously[3,5,21]. Smoking was not a significant risk factor for CCA, whereas alcohol consumption was in fact related to increased risk as we reported previously for subjects (and matched controls) recruited from another part of northern Thailand[5]. In the present study, control subjects were persons seeking a health check-up and thus possibly may have led a relatively healthier lifestyle compared with our experimental subjects. However, the discordance between the findings on smoking and alcohol drinking of the two studies are not yet explained. Although we did not find any significant association between CCA risk and polymorphisms in inflammation-related genes (*IL-6*, *IL-10*, *NF-kB*) or in drug metabolism-related genes (*CYP2E1*, *GSTT1* and *GSTM1*), persons with the *GSTT1* wild-type plus *CYP2E1* c1/c2 + c2/c2 genotype had a 3-fold greater risk than persons having the *GSTT1* wild-type plus *CYP2E1* c1/c1 wild-type genotype that was used as the reference. Hayashi *et al*[22] reported that the c2/c2 homozygous sequence placed upstream of the SV40 promoter and *chloramphenicol acetyltransferase* gene enhanced the expression of that gene, and the enhancement of expression by the c2/c2 sequence was about 10-fold that by the c1/c1 sequence. Thus, it is possible that *CYP2E1* is expressed at a higher level in the presence of the c2/c2 sequence than in the presence of the c1/c1 sequence, which is consistent with the known function of *CYP2E1* as a carcinogen-activating enzyme[23,24]. Because the numbers of cases and controls were not large, the conclusions from this work should be confirmed in a future study with

more cases and controls. In addition, genes encoding other drug-metabolizing enzymes should also be tested with respect to gene-gene interactions.

CONCLUSION

The presence of anti-OV in serum was associated with a 7- to 11-fold increased risk for CCA independently of the genetic polymorphisms of carcinogen-metabolizing and inflammation-related genes. While any single polymorphism was not significantly associated with CCA risk, persons with the *GSTT1* wild-type and *CYP2E1* c1/c2 + c2/c2 genotype had a 3-fold increased risk as compared with persons having the *GSTT1* wild-type and *CYP2E1* c1/c1 wild genotype. In addition to infection with OV, gene-gene interactions may be considered as one of the risk factors for CCA development.

ARTICLE HIGHLIGHTS

Research background

Cholangiocarcinoma (CCA) is a cancer of the hepatobiliary tract, and its incidence is extremely high in northeastern Thailand. This is related to the lifestyle of the inhabitants of this area consuming often raw fish, which carries the risk of ingesting fish-borne parasites, *Opisthorchis viverrini* (OV), a known CCA risk factor. While infection with OV has been listed as a carcinogen to humans by The International Agency for Research on Cancer, the parasitic infection alone is not sufficient to develop CCA; in fact, co-administration of a chemical carcinogen such as *N*-nitrosodimethylamine is necessary to induce CCA in animal model. In addition, genetic background related to the activation or detoxification of chemical carcinogens is reported to be involved in CCA risk. Also, elevated plasma IL-6 was associated with increased risk of CCA in patients infected with OV.

Research motivation

We already reported that infection with OV and genetic polymorphism of a drug-metabolizing enzyme gene, namely *GSTM1*, is related to CCA risk and that the combined effect of polymorphisms of the genes *8-oxoguanine glycosylase 1* and *GSTM1* is also relevant CCA risk in northeastern Thailand. In the present study, we further investigated possible associations of maintenance of chronic infection, exposure to a chemical carcinogen(s) and genetic background with CCA risk in northeastern Thailand.

Research objectives

To examine relations between risk for CCA and genetic polymorphisms in carcinogen-metabolizing (*CYP2E1*, *GSTT1* and *GSTM1*) and inflammation-related genes (*IL-6*, *IL-10* and *NF-κB*), and potential interactions among genetic polymorphisms of these genes on the CCA risk.

Research methods

All cases with CCA were identified between 1999 and 2005 upon a visit to the Ubon Ratchathani Cancer Centre in the northeastern province of Thailand. This hospital-based case-control study enrolled 95 case-control pairs matched by age (± 5 years) and sex. We examined relations between risk for CCA and genetic polymorphisms in carcinogen-metabolizing and inflammation-related genes, serum anti-OV, alcohol consumption, and smoking. Smoking and alcohol consumption status were ascertained using a structured questionnaire used in previous studies. Conditional logistic regression was employed to estimate CCA risk as OR due to each of genetic polymorphisms and possible interactions of those polymorphisms.

Research results

Although any single polymorphism was not significantly associated with CCA risk, persons with the *GSTT1* wild-type and *CYP2E1* c1/c2 + c2/c2 genotype had a 3-fold increased risk as compared with persons having the *GSTT1* wild-type and *CYP2E1* c1/c1 wild genotype. The presence of anti-OV in serum was associated with a 7- to 11-fold increased risk for CCA independently of the genetic polymorphisms of carcinogen-metabolizing and inflammation-related genes.

Research conclusions

An inflammatory condition produced by infection with OV indicated as raised anti-OV in serum has been associated with CCA risk. Our study added the finding that persons with the *GSTT1* wild-type and *CYP2E1* c1/c2 + c2/c2 genotype had a 3-fold increased risk for developing CCA. Therefore, both gene-gene interactions and OV infection should be considered as risk factors for cholangiocarcinogenesis in northeastern Thailand.

Research perspectives

CCA is still an intractable cancer. While our study revealed the interaction of polymorphisms of *GSTT1* and *CYP2E1* possibly contributes to development of CCA, the numbers of cases and controls were not large. The conclusions from this work should be confirmed in a future study with more cases and controls. In addition, genes encoding other drug-metabolizing enzymes should also be tested with respect to gene-gene interactions.

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FOOTNOTES

Author contributions: Miwa M secured funds and started the collaborative study in 1998 with the late Srivatanakul P who had been Thai side's active organizer based on the National Cancer Institute, Bangkok, until her death in 2020; Viwatthanasittiphong C, Muangphot M, Chenvidhya D, Jedpiyawongse A, Sripa B, Honjo S, and Miwa M designed and conducted the epidemiological study; Sripa B measured anti-OV and providing microbiological advice; You G, Zeng L, Tanaka H, Ohta E, Fujii T, Ohshima K, Tanaka M, Hamajima N performed analyses concerning genetic polymorphisms; You G, Zeng L, Miwa M and Honjo S conducted statistical analyses and prepared the manuscript; All authors have read and approved the final manuscript.

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Observational Study

Novel CABIN score outperforms other prognostic models in predicting in-hospital mortality after salvage transjugular intrahepatic portosystemic shunting

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Abstract

BACKGROUND

Transjugular intrahepatic portosystemic shunt (TIPS) is now established as the salvage procedure of choice in patients who have uncontrolled or severe recurrent variceal bleeding despite optimal medical and endoscopic treatment.

AIM

To analysis compared the performance of eight risk scores to predict in-hospital mortality after salvage TIPS (sTIPS) placement in patients with uncontrolled variceal bleeding after failed medical treatment and endoscopic intervention.

METHODS

Baseline risk scores for the Acute Physiology and Chronic Health Evaluation (APACHE) II, Bonn TIPS early mortality (BOTEM), Child-Pugh, Emory, FIPS, model for end-stage liver disease (MELD), MELD-Na, and a novel 5 category CABIN score incorporating Creatinine, Albumin, Bilirubin, INR and Na, were calculated before sTIPS. Concordance (C) statistics for predictive accuracy of in-hospital mortality of the eight scores were compared using area under the receiver operating characteristic curve (AUROC) analysis.

RESULTS

Thirty-four patients (29 men, 5 women), median age 52 years (range 31-80) received sTIPS for uncontrolled (11) or refractory (23) bleeding between August 1991 and November 2020. Salvage TIPS controlled bleeding in 32 (94%) patients with recurrence in one. Ten (29%) patients died in hospital. All scoring systems had a significant association with in-hospital mortality ($P < 0.05$) on multivariate analysis. Based on in-hospital survival AUROC, the CABIN (0.967), APACHE II (0.948) and Emory (0.942) scores had the best capability predicting mortality compared to FIPS (0.892), BOTEM (0.877), MELD Na (0.865), Child-Pugh (0.802) and MELD (0.792).

CONCLUSION

The novel CABIN score had the best prediction capability with statistical superiority over seven other risk scores. Despite sTIPS, hospital mortality remains high and can be predicted by CABIN category B or C or CABIN scores > 10 . Survival was 100% in CABIN A patients while mortality was 75% for CABIN B, 87.5% for CABIN C, and 83% for CABIN scores > 10 .

Key Words: Transjugular intrahepatic portosystemic shunt; Risk score; Portal hypertension; Variceal bleeding; Mortality

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Core Tip: This study compared the performance of a new CABIN score with seven existing risk scores to predict in-hospital mortality after salvage transjugular intrahepatic portosystemic shunt (TIPS) placement in 34 patients with uncontrolled variceal bleeding after failed medical treatment and endoscopic intervention. Using concordance statistics for predictive accuracy of in-hospital mortality the novel 5 category CABIN score incorporating Creatinine, Albumin, Bilirubin, INR and Na outperformed the APACHE II, BOTEM, Child-Pugh, Emory, FIPS, MELD and MELD-Na scores when compared by area under the receiver operating characteristic curve (AUROC) analysis. Survival was 100% in CABIN A patients while mortality was 75% for CABIN B, 87.5% for CABIN C, and 83% for CABIN scores > 10 .

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INTRODUCTION

Transjugular intrahepatic portosystemic shunt (TIPS) is now established as the salvage procedure of choice in patients who have uncontrolled or severe recurrent variceal bleeding despite optimal medical and endoscopic treatment[1]. Key clinical distinctions exist in the spectrum of patients undergoing TIPS, ranging from high-risk cirrhotic patients with liver decompensation and uncontrolled variceal bleeding necessitating an emergent salvage TIPS (sTIPS) to those with well-preserved liver function undergoing an elective TIPS for refractory bleeding. Current risk stratification of patients who have refractory variceal bleeding and require sTIPS is however imperfect. Although TIPS is a minimally invasive procedure, appropriate patient selection is crucial to identify patients who would benefit from the procedure, considering the substantial risks of hepatic encephalopathy, liver failure and increased overall morbidity and mortality in high-risk individuals[2,3].

Several prognostic and risk scores have been developed to identify patients at risk for a poor clinical outcome after sTIPS. These include the Acute Physiology and Chronic Health Evaluation (APACHE) II [4], Bonn TIPS early mortality (BOTEM)[5], Child-Pugh (C-P)[6], Emory[7], Freiburg index of post-TIPS survival (FIPS)[8], model for end-stage liver disease (MELD)[9], and Model for End-Stage Liver Disease sodium (MELD-Na)[10] scores. In this study the accuracy of a novel CABIN score, which was developed to overcome limitations of existing scoring systems, was compared to established risk scores for the prediction of in-hospital mortality following sTIPS.

MATERIALS AND METHODS

In this retrospective observational analysis, eight risk scores were evaluated in a cohort which included all adult patients who underwent sTIPS for uncontrollable or life-threatening refractory variceal

bleeding in the Surgical Gastroenterology Unit at Groote Schuur Hospital and the University of Cape Town Private Academic Hospital between August 1991 and November 2020. The study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for reporting observational studies[11]. Baseline demographic, clinical and endoscopic data and biochemical variables were collected on admission. The anonymized and de-identified information were retrieved from a prospectively maintained ethics approved registry for patients treated for esophageal varices (Table 1).

Details of the acute bleeding management protocol and the endoscopic interventional techniques used in our unit have been published previously[12-15]. In patients who had endoscopically uncontrolled bleeding a Minnesota balloon tube or a Danis esophageal stent (Ella-CS, Hradec Kralove, Czech Republic) was inserted to tamponade variceal bleeding and endotracheal intubation was used for airway protection when indicated[16]. In this high-risk group with uncontrolled variceal bleeding and those with refractory life-threatening bleeding despite endoscopic intervention and somatostatin infusion, sTIPS was performed as an emergency procedure under general anaesthesia with placement of an expandable uncovered 10 mm Wallstent (Boston Scientific, Marlborough, MA, United States)[15].

The study protocol followed the Baveno recommendations and defined uncontrolled or persistent variceal bleeding as the need for a transfusion of 4 units of blood or more within 6 h and the inability to achieve an increase in systolic blood pressure to 70 mmHg or more or a pulse reduction to less than 100/min. Contraindications to sTIPS in our unit were severe pulmonary hypertension, severe tricuspid regurgitation, congestive heart failure, fibropolycystic liver disease, uncontrolled systemic sepsis and unrelieved biliary obstruction. Relative contraindications were congenital hepatic fibrosis, portal vein thrombosis, obstruction of all hepatic veins and severe coagulopathy (INR > 5).

Details of the newly developed five component CABIN score are given in Supplementary Table 1. Each CABIN variable is scored from one to five and the cumulative total is calculated by adding the individual values of the five biochemical components (Creatinine, Albumin, Bilirubin, INR (international normalized ratio) and Na (sodium)). The best total CABIN score computes at 5 points and the worst at 25 points. Four CABIN categories (A-D) were established (A: 5-10 points, B: 11-15, C: 16-20, D: 21-25).

The CABIN score and seven previously described scoring systems, APACHE II, BOTEM, Child-Pugh, Emory, FIPS, MELD, and MELD-Na scores were calculated based on clinical evaluation and laboratory values obtained before the sTIPS procedure. The primary study outcome measure was prediction of in-hospital mortality after sTIPS and compared the relative performances of the seven established scoring models and the new CABIN score.

Statistical analysis

All clinical data and variables were collected and managed using the REDCap electronic data capturing software licensed to the University of Cape Town[17]. Statistical computations were made using IBM SPSS statistics (version 26.0, IBM, United States). Statistical significance was set at $P < 0.05$. Continuous data were reported as mean $\pm$ SD or medians and range and discrete data as percentages. To evaluate the performance of the various scoring systems to predict in-hospital mortality the concordance C-statistic [area under the curve (AUC) of the receiver operating characteristic (ROC) curves] was used.

Ethical considerations

The study protocol was approved by the Human Research Ethics Committee (HREC Ref No. 120/2019) of the University of Cape Town and the research was conducted in accordance with the Declaration of Helsinki.

RESULTS

A total of 564 patients with variceal bleeding were treated during the study period. In 530 patients (94%), bleeding was controlled by endoscopic intervention and medication. In 34 patients (6%) who constitute the study population and underwent sTIPS, bleeding was either uncontrollable *ab initio* ($n = 11$) or life-threatening refractory ($n = 23$) despite optimal endoscopic and pharmacological management.

The demographic and clinical data of the patients are summarized in Table 1. No patients had a concomitant HCC or portal vein thrombosis at the time of TIPS insertion. Before sTIPS 19 patients had a median of three (1-9) injection sclerotherapy treatment (IST) sessions and 20 had a median of two (1-6) endoscopic variceal ligation (EVL) sessions with a median of 10 bands placed per session. Five patients had both IST and EVL. Median units of blood transfused before sTIPS was six (3-12), and 14 patients required either Minnesota balloon tamponade ($n = 12$) or placement of a Danis stent ($n = 2$) for temporarily control of bleeding before the sTIPS procedure. Eleven patients required endotracheal intubation and mechanical ventilation and nine required inotropic support.

Technical success for sTIPS was 100% and therapeutic success (control of bleeding) was achieved in 31 of 34 (91%) patients. Bleeding persisted in two patients (6%) despite a patent sTIPS on repeat US-doppler examination and one patient developed recurrent bleeding in hospital during the index

Table 1 Demographic, clinical characteristics and risk prediction scores of 34 patients undergoing salvage transjugular intrahepatic portosystemic shunt, *n* %

Variable	Total cohort (<i>n</i> = 34)	Survived (<i>n</i> = 24)	In-hospital death (<i>n</i> = 10)	<i>P</i> value
Demographics				
Age (mean ± SD)	52 ± 11.6	50 ± 10.5	57 ± 12.9	0.107
Sex				
Male	29 (85)	22 (92)	7 (70)	0.104
Female	5 (15)	2 (8)	3 (30)	
Cause of cirrhosis				
Alcohol related	22 (65)	15 (63)	7 (70)	0.938
Non-alcohol related	12 (35)	9 (37)	3 (30)	
Child-Pugh grade				
A	3 (9)	3 (12)	0	0.022
B	19 (56)	16 (67)	3 (30)	
C	12 (35)	5 (20)	7 (70)	
Risk prediction scores				
APACHE II (mean ± SD)	13.4 ± 4.7	11.4 ± 3.3	18.3 ± 3.8	0.196
BOTEM (mean ± SD)	5.4 ± 1.1	5.0 ± 0.9	6.3 ± 0.7	0.964
CABIN (mean ± SD)	10.9 ± 5.0	8.3 ± 1.8	17.0 ± 3.8	0.133
CHILD-PUGH (mean ± SD)	8.9 ± 1.8	8.2 ± 1.8	10.6 ± 2.0	0.001
EMORY (mean ± SD)	3.2 ± 0.9	2.8 ± 0.7	4.3 ± 0.5	0.497
FIPS (mean ± SD)	-0.3 ± 0.9	-0.6 ± 0.9	0.5 ± 0.5	0.205
MELD (mean ± SD)	15.0 ± 6.2	13 ± 4.8	19.8 ± 6.7	0.007
MELD-Na (mean ± SD)	16.9 ± 7.4	14 ± 5.3	23.9 ± 7.1	< 0.001

SD: Standard deviation; APACHE II: Acute physiology and chronic health evaluation II; BOTEM: Bonn TIPS early mortality; CABIN: Creatinine, Albumin, Bilirubin, INR, Sodium score; FIPS: Freiburg index of post-TIPS survival; MELD: Model for end-stage liver disease; MELD-Na: Model for end-stage liver disease sodium.

admission after initial control of bleeding by sTIPS.

Ten patients (29.4%) died in hospital at a median of 5 d following the procedure (range 1-10 d) of progressive liver failure (*n* = 4), MOF (2), alcoholic cardiomyopathy (*n* = 2) or uncontrolled variceal bleeding (*n* = 2). Mortality in C-P grade A patients was 0%, in C-P grade B patients 16% and C-P grade C patients 58%. In patients who died the median C-P score was 11, (range 7-13), median MELD score was 18 (range 11-29) and median MELD Na score was 25 (range 11-33). Nine of the 12 (75%) patients who required pre-sTIPS balloon tamponade died, while all nine (100%) patients who were hypotensive (systolic blood pressure < 70 mmHg) and with the combination of > 8 unit blood transfusion, inotropic support, balloon tamponade and mechanical ventilation died.

The two patients with persistent bleeding after TIPS underwent repeat endoscopy and ultrasound-guided Histoacryl and coil injection of residual gastric varices with resolution. The patient with recurrent bleeding in hospital underwent a gastric devascularization for control of gastric varices.

Figure 1 shows the graphic representation of the comparative performances of the eight risk scores in predicting in-hospital death following sTIPS. The CABIN score (AUROC 0.967) had the highest discriminative ability in predicting in-hospital death compared to the APACHE II (AUROC 0.948), BOTEM (AUROC 0.877), C-P (AUROC 0.802), EMORY (AUROC 0.942), FIPS (AUROC 0.892), MELD (AUROC 0.792), and MELD-Na (AUROC 0.865) scores as detailed in Table 2. The median CABIN score in the 24 in-hospital TIPS survivors was 8 (range 5-18) compared to a median of 17 (range 11-22) in the 10 deaths. CABIN A patients had a 100% survival, compared to 25% and 12.5% survival in CABIN B and CABIN C category patients respectively. CABIN points of 11 or more provided a clear survival cut-off. No patients with CABIN scores < 10 died while 83% of patients with CABIN scores of > 11 died.

Table 2 Performance of various risk prediction scores in predicting in-hospital death following salvage transjugular intrahepatic portosystemic shunt

	In-hospital deaths			
	AUC	STD error	P value	95% confidence interval
APACHE II	0.948	0.035	0	0.879–1.000
BOTEM	0.877	0.059	0.001	0.762–0.992
CABIN	0.967	0.028	0	0.912–1.000
CHILD-PUGH	0.802	0.084	0.006	0.638–0.967
EMORY	0.942	0.038	0	0.868–1.000
FIPS	0.892	0.055	0	0.783–1.000
MELD	0.792	0.082	0.008	0.631–0.952
MELD Na	0.865	0.077	0.001	0.713–1.000

AUC: Area under the curve; SD: standard deviation; APACHE II: Acute physiology and chronic health evaluation II; BOTEM: Bonn TIPS early mortality; CABIN: Creatinine albumin bilirubin INR sodium score; FIPS: Freiburg index of post-TIPS survival; MELD: Model for end-stage liver disease; MELD-Na: Model for end-stage liver disease sodium.

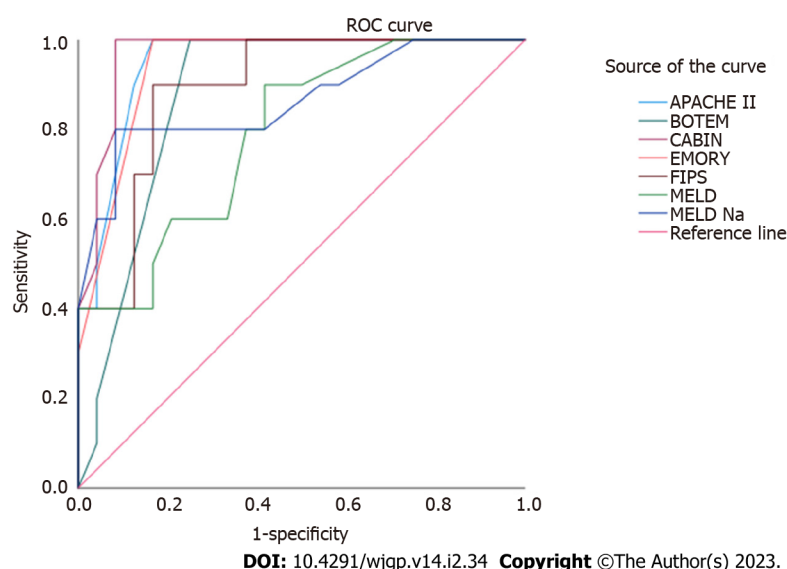


Figure 1 Performance of various risk prediction scores in predicting in-hospital death following salvage transjugular intrahepatic portosystemic shunt. APACHE II: Acute physiology and chronic health evaluation II; BOTEM: Bonn TIPS early mortality; CABIN: Creatinine, Albumin, Bilirubin, INR, Sodium score; FIPS: Freiburg index of post-TIPS survival; MELD: Model for end-stage liver disease; MELD-Na: Model for end-stage liver disease sodium; TIPS: Transjugular intrahepatic portosystemic shunt; ROC: Receiver operating characteristic.

DISCUSSION

The unique safety profile and minimally invasive characteristics conferred by TIPS provide an effective reduction in portal pressure and make the procedure the ideal rescue intervention for variceal bleeding not controlled by endoscopic intervention and pharmacological therapy[18]. In this study we compared the relative performances of eight scoring models, including the novel CABIN score, in predicting in-hospital mortality in a high-risk cohort of patients who underwent sTIPS placement. Although sTIPS controlled variceal bleeding in 94% of patients, over-all in-hospital mortality was 29.4% and increased exponentially in those who required > 8 unit blood transfusion, inotropic support, esophageal balloon tamponade and mechanical ventilation. Log-rank comparisons of survival curves showed that of the eight scores evaluated, the CABIN, APACHE II and Emory scores had the highest AUROC values and the best discriminatory ability with C-statistic values all exceeding 0.9. Of these three top contenders, the CABIN score (0.967) had the best discriminatory and predictive capability. As a corollary, this study also demonstrates the predictive ability of the CABIN score with 100% survival observed in patients in the CABIN A category (< 10 points) after sTIPS.

The reported mortality rate after TIPS placement varies widely due to differing inclusion criteria, timing of TIPS placement, the spectrum and severity of the underlying liver disease and inclusion in some reports of patients with active bleeding during urgent TIPS as well as stable patients undergoing elective TIPS[19-21]. In the 22 studies exclusively reporting salvage or rescue TIPS in patients with uncontrolled life-threatening or endoscopically unmanageable variceal bleeding, as in this study, in-hospital mortality rates range from 17% to 56% which are significantly higher than for elective TIPS[22-43] (Table 3). Accurate prediction of outcome following sTIPS is thus a crucial element of management and the optimal prognostic score should ideally be able to distinguish two groups, patients with a better prognosis and likely to survive and those with a high or prohibitive risk of death.

Most of the current prognostic scores used in sTIPS patients have intrinsic limitations due to the selection and weighting of the constituent components. The MELD score, which was initially created to predict survival following elective placement of TIPS, is currently the most widely used liver-related prognostic score both in clinical practice and research and especially as a tool for organ allocation[9]. Although the MELD score was a prospectively developed and validated indicator of the severity of end-stage liver disease that utilizes quantitative and objective measures, including bilirubin, creatinine and INR values, the score has potential limitations. A further caveat is the maximum assigned value of serum creatinine which is capped at four even when the measured serum level is higher. Modifications to overcome MELD shortcomings have included reweighting the model's coefficients, altering the laboratory components and the addition of new variables including serum sodium ('MELD-Na'), albumin [termed '5-variable MELD' (5vMELD)][44] and female gender (MELD 3.0)[45]. These modifications are more discriminative than either MELD or MELD-Na in transplant assessment and use similar elements as the CABIN score.

The inclusion of subjective clinical components in other proposed prognostic models may also limit precision and reproducibility of score assignments. The C-P, Emory and BOTEM scores all have at least one component that may be perceived as subjective while the APACHE II and BOTEM scores lack specificity for liver disease which limits their capacity to predict outcomes after liver interventions such as sTIPS. In addition the C-P, Emory, and BOTEM scores are limited by a ceiling effect in which laboratory values above a particular cut-off level are not distinguished from one another in terms of higher scoring[4-7,9,10]. The FIPS overcomes some of these limitations by using four objective components, age, bilirubin, albumin, and creatinine levels[8].

In a meta-analysis, which included 11 studies and 2037 patients Zhou *et al*[46] found that MELD was superior to the C-P score in predicting 3-mo survival after TIPS but not 1-mo, 6-mo or 12-mo survival. Zhang *et al*[47] found that C-P grade C and MELD > 10 but not the Emory, BOTEM or SB/PLT scores were predictors of survival in Chinese cirrhotic patients treated with TIPS. Gaba *et al*[48] reported that MELD and MELD-Na scores had the best capability to predict early mortality in an American population compared with bilirubin and the C-P, Emory, PI, APACHE II, and BOTEM scores. In a comparison of the MELD, C-P and Emory scores Schepke *et al*[49] found that all three models predicted 3-mo survival with similar accuracy, but the MELD score was marginally superior to the C-P score for both 12- and 36-mo survival. In patients with refractory variceal bleeding Rubin *et al*[39] found that survival was inversely proportional to C-P class and APACHE II scores. The single determinant most closely associated with decreased survival in the first month following TIPS was the APACHE II score, with a score of 18 stratifying patients into low and high mortality risk groups (Table 3). Only one of 13 patients with C-P class C cirrhosis and an APACHE II score exceeding 18 survived > 30 d[39]. In the Hermie study early mortality was associated with a MELD score of at least 19 and hemodynamic instability at the time of admission[32] (Table 3). If hemodynamic instability was combined with a high MELD score, the 6-week mortality peaked at 77.8%[32]. In a multicentre French study Walter *et al*[50] reported that sTIPS mortality was > 90% in patients who had lactate levels ≥ 12 mmol/L and/or a MELD score ≥ 30 .

In view of these differing outcomes, the development of a prognostic model to accurately stratify the risk profile of patients undergoing sTIPS may be invaluable in guiding treatment. The novel CABIN score used in this study was developed as a point-based tool to improve prognostic prediction specifically for patients undergoing emergent sTIPS and circumvents the complex computations of the MELD and other scores. This new score avoids subjective elements and can be calculated at the bedside providing a refined, granular grading system from a minimal laboratory dataset with scores ranging from 5 to 25. The CABIN score achieved significant prognostic discrimination reflected by in-hospital survival of 100% in patients in the CABIN A category (5-10 points), while patients in the CABIN B category (11-15) score had a 25% and those in the CABIN C category (16-20) a 12.5% survival. Our model predicted in-hospital mortality with high accuracy and showed statistical superiority over the other seven contenders, including MELD and C-P scores. Moreover, of all the examined models, only the CABIN, APACHE II and Emory scores exceeded a C-statistic value of 0.9.

There are inevitable and specific limitations to our study. Firstly, this investigation is limited by its small sample size, retrospective design, and lack of a control group. Secondly, the study has a clear selection bias which restricts universal applicability as these patients were treated in a single, well-resourced tertiary care referral center with round the clock skilled endoscopic and TIPS access. Thirdly, because patients were accrued over three decades, technical differences in TIPS placement and improvements in medical care during the study period would have contributed to differences in clinical

Table 3 Published series of salvage transjugular intrahepatic portosystemic shunt for uncontrolled variceal bleeding

Ref.	Country	No. of patients	C-P grade A/B/C	Initial control of bleeding %	30-d mortality %	Persistent/Recurrent rebleeding	Survival %	Prognostic factors
Azoulay <i>et al</i> [22], 2001	France	58	3/8/47	90	29	17	51.7 (12 mo)	Sepsis, vasoactive drugs, balloon tamponade
Bañares <i>et al</i> [23], 1998	Spain	56	11/22/23	95	28	22 (1 mo)	72 (30 d)	Ascites, HE, albumin
Barange <i>et al</i> [24], 1999	France	32	3/14/15	90	25	14	75 (30 d)	ND
Bizollon <i>et al</i> [25], 2001	France	28	0/11/17	96	25 (40 d)	18	52 (2 yr)	↑Creatinine, ↑bilirubin
Casadaban <i>et al</i> [26], 2015	United States	101	2/46/52	89	31	21	44 (12 mo)	↑Bilirubin, ↑creatinine, ↑INR, non-alcoholic liver disease
Chau <i>et al</i> [27], 1998	England	84	4/17/63	98	34	30 (30 d)	66 (30 d)	ND
Encarnacion <i>et al</i> [28], 1995	United States	64	2/32/31	98	19	29 (6 mo)	56 (12 mo)	Haemodynamic instability
Gazzera <i>et al</i> [29], 2012	Italy	82	ND	94	25.6	13.4	74.4 (30d)	Child-Pugh C, ↑creatinine, ↑PT
Gerbes <i>et al</i> [30], 1998	Germany	11		91	27	27	73 (12 mo)	ND
Helton <i>et al</i> [31], 1993	United States	23	0/15/18	74	56 (in hospital)	39	ND	Emergency TIPS, active bleeding
Hermie <i>et al</i> [32], 2018	Belgium	32	ND/ND/14	97	31	0	69	MELD > 19, Haemodynamic instability
Jabbour <i>et al</i> [33], 1996	United States	25	ND/ND/8	96	44	ND	56 (30 d)	Child-Pugh C, urgent TIPS
Jalan <i>et al</i> [34], 1995	Scotland	19	3/3/13	100	42	15.6	58 (30 d)	Liver failure, sepsis
Maimone <i>et al</i> [36], 2019	England	144	11/55/78	ND	36 (6 wk)	29	64 (6 wk)	↑MELD, ↑Child-Pugh score
Le Moine <i>et al</i> [35], 1994	Belgium	24	3/13/9	96	17	25	29 (5 mo)	ND
McCormick <i>et al</i> [37], 1994	England	20	1/7/12	100	60 (40 d)	40	30	ND
Patch <i>et al</i> [38], 1998	England	54	5/20/29	91	48 (6 wk)	11	53 (6 mo)	Ventilation, ↑WBC, platelets, ↑creatinine
Rubin <i>et al</i> [39], 1995	United States	49	3/23/23	84	40%	16	ND	C-P grade C, APACHE II > 18
Sanyal <i>et al</i> [40], 1996	United States	30	1/7/22	100	37	7	60 (6 wk)	> 70 yr, bilirubin > 6 mg/dL, creatinine > 3 mg/dL, HE, ARDS
Tyburski <i>et al</i> [41], 1997	United States	33	0/5/28	ND	27	15	58 (12 mo)	Albumin < 2.5 g/dL, bilirubin > 3 mg/dL, PT > 15 s
Tzeng <i>et al</i> [42], 2009	Taiwan	107	ND	ND	28	ND	50 (12 mo)	C-P score > 11, MELD > 20
Zhu <i>et al</i> [43], 2019	China	58	5/36/7	91.2	12.3 (6 wk)	10.5 (6 wk)	81.8 (12 mo)	Ventilation, ICU

APACHE: Acute Physiology and Chronic Health Evaluation; ARDS: Adult Respiratory Distress Syndrome; C-P: Child-Pugh; INR: International normalized ratio; TIPS: Transjugular intrahepatic portosystemic shunt; MELD: Model for end-stage liver disease; HE: Hepatic encephalopathy; ND: No data; ICU: Intensive care unit; WBC: White blood cells.

outcomes over time. Fourthly, this new score has been developed using a derivation dataset and requires confirmation and external validation in a similar sTIPS patient group. The robustness of this study is enhanced by the prospective data collection, supervision by the same investigators during the study period, restriction of subjects to a well-defined cohort of cirrhotic patients with uncontrolled exsanguinating bleeding and complete follow-up. The use of all-cause mortality as the primary outcome provided a consistent and objective end point.

CONCLUSION

In conclusion, the novel CABIN prognostic score, which is objective, quantitative, and reproducible, combines five easily obtained laboratory test results and provides improved statistical power predicting in-hospital mortality in patients with uncontrolled variceal bleeding undergoing sTIPS. The CABIN score identified high-risk patients and outperformed other scoring systems in predicting in-hospital mortality. Despite the fact that mortality was 75% for CABIN B, 87.5% for CABIN C, and 83% for CABIN scores > 10 in this study, this high-risk category should not be denied consideration for an emergency TIPS and should be assessed on a case-by-case basis especially in units where there is prompt access to liver transplantation after sTIPS. This study was based on a small defined cohort of predominantly alcoholic decompensated cirrhotic patients undergoing emergent TIPS and this newly developed derivative CABIN score will need further prospective external validation before being considered for general clinical application.

ARTICLE HIGHLIGHTS

Research background

Transjugular intrahepatic portosystemic shunt (TIPS) is now established as the salvage procedure of choice in patients who have uncontrolled or severe recurrent variceal bleeding despite optimal medical and endoscopic treatment.

Research motivation

Although TIPS is a minimally invasive procedure, appropriate patient selection is crucial to identify patients who would benefit from the procedure, considering the substantial risks of hepatic encephalopathy, liver failure and increased overall morbidity and mortality in high-risk individuals.

Research objectives

In this study the accuracy of a novel CABIN score, which was developed to overcome limitations of existing scoring systems, was compared to established risk scores for the prediction of in-hospital mortality following sTIPS.

Research methods

Eight risk scores were evaluated in a cohort which included all adult patients who underwent sTIPS for uncontrollable or life-threatening refractory variceal bleeding. A new five component CABIN score was devised in which each CABIN variable was scored from one to five and the cumulative total is calculated by adding the individual values of the five biochemical components (Creatinine, Albumin, Bilirubin, INR (international normalized ratio) and Na (sodium)). The best total CABIN score computes at 5 points and the worst at 25 points. Four CABIN categories (A-D) were established (A: 5-10 points, B: 11-15, C: 16-20, D: 21-25). The CABIN score and seven previously described scoring systems, Acute Physiology and Chronic Health Evaluation (APACHE) II, Bonn TIPS early mortality (BOTEM), Child-Pugh, Emory, FIPS, model for end-stage liver disease (MELD), and MELD-Na scores were calculated based on clinical evaluation and laboratory values obtained before the sTIPS procedure. The primary study outcome measure was prediction of in-hospital mortality after sTIPS and compared the relative performances of the seven established scoring models and the new CABIN score.

Research results

In 34 patients (6%) who underwent sTIPS, bleeding was either uncontrollable *ab initio* ($n = 11$) or life-threatening refractory ($n = 23$) despite optimal endoscopic and pharmacological management. Ten patients (29.4%) died in hospital at a median of 5 d following the procedure (range 1-10 d). Nine of the 12 (75%) patients who required pre-sTIPS balloon tamponade died, while all nine (100%) patients who were hypotensive (systolic blood pressure < 70 mmHg) and with the combination of > 8 unit blood transfusion, inotropic support, balloon tamponade and mechanical ventilation died. The CABIN score [area under the receiver operating characteristic curve (AUROC) 0.967] had the highest discriminative ability in predicting in-hospital death compared to the APACHE II (AUROC 0.948), BOTEM (AUROC

0.877), C-P (AUROC 0.802), EMORY (AUROC 0.942), FIPS (AUROC 0.892), MELD (AUROC 0.792), and MELD-Na (AUROC 0.865) scores. The median CABIN score in the 24 in-hospital TIPS survivors was 8 (range 5-18) compared to a median of 17 (range 11-22) in the 10 deaths. CABIN A patients had a 100% survival, compared to 25% and 12.5% survival in CABIN B and CABIN C category patients respectively. CABIN points of 11 or more provided a clear survival cut-off. No patients with CABIN scores < 10 died while 83% of patients with CABIN scores of > 11 died.

Research conclusions

The novel CABIN prognostic score, which is objective, quantitative, and reproducible, combines five easily obtained laboratory test results and provides improved statistical power predicting in-hospital mortality in patients with uncontrolled variceal bleeding undergoing sTIPS. The CABIN score identified high-risk patients and outperformed other scoring systems in predicting in-hospital mortality. Despite the fact that mortality was 75% for CABIN B, 87.5% for CABIN C, and 83% for CABIN scores > 10 in this study, this high-risk category should not be denied consideration for an emergency TIPS and be assessed on a case by case basis especially in units where there is prompt access to liver transplantation after sTIPS.

Research perspectives

This study was based on a small defined cohort of predominantly alcoholic decompensated cirrhotic patients undergoing emergent TIPS and this newly developed derivative CABIN score will need further prospective external validation before being considered for general clinical application.

FOOTNOTES

Author contributions: Krige J, Jonas E and Robinson C designed the research study; Krige J, Jonas E, Robinson C and Kotze U collected the data and performed the research; Krige J, Jonas E, Robinson C, Kotze U, Beningfield S, Bernon M, Burmeister S, and Kloppers C analyzed the data and wrote the manuscript; All authors have read and approve the final manuscript.

Institutional review board statement: The study protocol was approved by the Human Research Ethics Committee (HREC Ref No. 120/2019) of the University of Cape Town and the research was conducted in accordance with the Declaration of Helsinki.

Informed consent statement: Since this was a retrospective observational study using existing anonymized data, the requirement for informed consent from the study participants was waived by the Institutional Review Board.

Conflict-of-interest statement: All the authors declare no conflict of interest.

Data sharing statement: Dataset available from the corresponding author at jej.krige@uct.ac.za.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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REVIEW

- 46 Role of p53 suppression in the pathogenesis of hepatocellular carcinoma

Choudhary HB, Mandlik SK, Mandlik DS

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Role of p53 suppression in the pathogenesis of hepatocellular carcinoma

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Abstract

In the world, hepatocellular carcinoma (HCC) is among the top 10 most prevalent malignancies. HCC formation has indeed been linked to numerous etiological factors, including alcohol usage, hepatitis viruses and liver cirrhosis. Among the most prevalent defects in a wide range of tumours, notably HCC, is the silencing of the p53 tumour suppressor gene. The control of the cell cycle and the preservation of gene function are both critically important functions of p53. In order to pinpoint the core mechanisms of HCC and find more efficient treatments, molecular research employing HCC tissues has been the main focus. Stimulated p53 triggers necessary reactions that achieve cell cycle arrest, genetic stability, DNA repair and the elimination of DNA-damaged cells' responses to biological stressors (like oncogenes or DNA damage). To the contrary hand, the oncogene protein of the murine double minute 2 (MDM2) is a significant biological inhibitor of p53. MDM2 causes p53 protein degradation, which in turn adversely controls p53 function. Despite carrying wt-p53, the majority of HCCs show abnormalities in the p53-expressed apoptotic pathway. High p53 *in-vivo* expression might have two clinical impacts on HCC: (1) Increased levels of exogenous p53 protein cause tumour cells to undergo apoptosis by preventing cell growth through a number of biological pathways; and (2) Exogenous p53 makes HCC susceptible to various anticancer drugs. This review describes the functions and primary mechanisms of p53 in pathological mechanism, chemoresistance and therapeutic mechanisms of HCC.

Key Words: Hepatocellular carcinoma; p53; Tumour suppressor gene; Murine double minute 2; Chemoresistance

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Core Tip: This review articles covers all the points related to p53 gene suppression in the pathogenesis of hepatocellular carcinoma. The review manuscript also includes information about tumour suppressor P53, pathogenesis of P53-associated hepatocellular carcinoma (HCC), role of P53 in mechanisms of hepatocarcinogenesis, roles of P53 in extrinsic factor-induced liver carcinogenesis, relationship between hepatitis C virus core protein and P53 in HCC, other mechanisms of progression to cirrhosis and HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most prevalent form of primary liver cancer and typically brought on by chronic liver diseases. HCC every year has 841000 new cases and 781000 deaths which makes HCC the fourth leading cause of cancer-related death globally[1]. By 2025 the annual diagnosed cases of HCC are expected to outstretch up to 1 million. HCC can appear on its own or more usually results from chronic liver illnesses of various etiological factors, like cirrhosis, alcoholic steatohepatitis (ASH) and non-ASH (NASH), viral infections [hepatitis C virus (HCV) and hepatitis B virus (HBV)] and hepatitis B and C[2] (Figure 1). As transplantation, ablation and resection can only be used for the treatment of early-stage HCC patients and since the majority of patients are diagnosed with severe stages or intrinsic chronic hepatic disease, discovering novel therapies to increase survival time and lower the likelihood of tumour recurrence is an important objective in the domain of HCC. According to the Barcelona clinic liver cancer staging system, Sorafenib has been utilized for several years as a treatment for HCC that has progressed to an advanced stage[2]. Nevertheless, concerns regarding its effectiveness and harmful effects have been raised[3], including the activation of metabolizing enzymes [4]. While the use of immunotherapy has altered the approach to treating advanced HCC, not all patients with HCC respond positively to these recent therapies. Consequently, there are ongoing investigations into various strategies to enhance the effectiveness of HCC medication by incorporating chemosensitizers[5] and/or personalized combination treatments[6]. Additionally, patients who receive immunotherapy may experience an upsurge in immune-related adverse events[7]. In this situation, tailoring HCC therapy to each patient is becoming an essential priority in order to achieve the greatest possible clinical advantage. Along with this, there is a need to find new biomarkers that can identify potential responders to pharmacological treatment by accurately predicting the clinical response[2].

The wild-type protein of the tumour suppressor gene p53 is responsible for regulating the cell cycle and inducing apoptosis after DNA damage[8]. However, if p53 undergoes a mutation, cells with DNA damage can avoid apoptosis and develop into cancerous cells[9]. Additionally, the mutant p53 protein, which loses the function of the wild-type protein, can amass in the nuclei of cells and is considered to be a highly specific marker for malignancy[10]. Studies have indicated that alterations in p53 are linked to tumour differentiation, vascular invasion, tumour stage, Child-Pugh class and serum alpha fetoprotein in HCC[11]. Nevertheless, the prognostic significance of p53 alterations in HCC is not yet fully understood and conflicting results have been reported in various studies[8]. While some studies have suggested that tumour p53 upregulation and serum anti-p53 antibody elevation are linked to recurrence-free survival and overall survival in HCC patients, other studies have not confirmed these findings[12]. Furthermore, most studies have indicated that HCC patients with a mutant p53 phenotype experience poor survival, but the prognostic effect has varied widely with a broad range of hazard ratios due to small and diverse studies[13].

Despite the progress made in treating certain other types of cancer, there isn't any effective treatment available for HCC and presently, chemotherapy and surgical resection are the two most common treatment options[14]. As a result, learning more about the molecular basis for the etiology of HCC may help researchers to find novel therapeutic approaches[14]. This review seeks to examine the biological mechanisms implicated in development of HCC, with a specific focus upon the murine double minute 2 (MDM2)-p53 networks and the hypothesised function of MDM2 in suppressing p53 expression. By knowing how MDM2 contributes in the development of HCC, we may potentially find methods to inhibit HCC as well as discover novel therapeutic approaches[14]. We also explore the function of cellular biology in identifying novel HCC biomarkers, utilizing MDM2-p53 linkages as prognostic indicators and considering future treatment strategies for HCC[14].

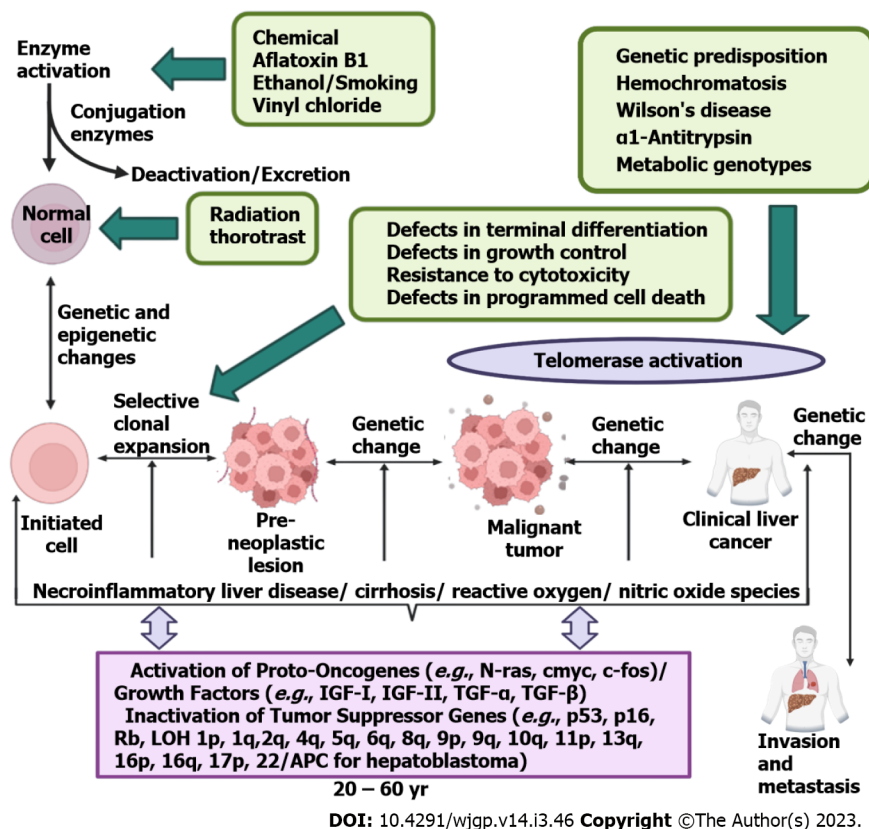


Figure 1 Etiological factors of hepatocellular carcinoma. Aflatoxin B1, vinyl chloride, smoking, ethanol, and many such factors leads to the development of hepatocellular carcinoma (HCC). Genetic predisposition, hemochromatosis and metabolic genotypes could also be the reason for the initiation of the HCC. There are many stages in the development of HCC including formation of pre-neoplastic lesion and malignant tumour (Created with BioRender.com). IGF: Insulin-like growth factor; TGF- α : Tumour growth factor- α ; TGF- β :

TUMOUR SUPPRESSOR P53

Nuclear protein that is produced by the human p53 gene is essential for cell division. It takes up roughly 20 kb of DNA. It was located on the short arm of chromosome 17 (17 p. 13.1) *via* southern filter hybridization of DNA from a hybrid of human-rodent[15]. There are 11 exons in the gene. The human p53 protein has 4 primary functional domains and 393 amino acids, according to earlier studies. There are more discussions of the p53 gene's and protein's molecular structure in the literature[16]. The discovery that Simian virus 40 (SV40)-transformed cells produce novel protein type which might be extracted by serum of anti-SV40 tumour led to the detection of the p53 protein, a protein having a noticeable molecular mass of 53 kDa[17]. Additional research showed that fluorophenylalanine therapy of SV40-infected monkey cells resulted in an increment in cellular thymidine absorption and a progressive suppression of viral and host DNA synthesis[14]. Transformed mouse cells, such as leukemias, murine sarcoma virus and spontaneously transformed fibroblasts, have been shown to express p53 in various cellular studies[18,19]. *In-vitro* virus sequence-specific DNA linkage is blocked by the wild-type p53 protein's complex formation with the hepatitis B virus X protein (HBx). The broad range of p53 function changes caused by HBx contributes to the emergence of human HCC[20]. The discovery of p53 in human leukaemia cells and transformed cell lines raises the possibility that p53 might influence the gene makeup of some types of leukaemia[21]. The production of p53 protein seems towards being essential for the mechanism of physiological cycling cells, in addition to its control over how dormant normal cells move from the G0-phase to the S-phase of the cell cycle. While p53 in experimentally altered (Meth A) mice cells appeared to have entered the stage of translation of RNA, transcription of p53 in growth inhibited healthy cells is regulated at the transcriptional or post-transcriptional stage[22]. Additionally, p53 takes involvement in the conversion of healthy embryonic cells and can work alongside Ha-ras oncogene to accomplish this[23]. In untransformed cells, it displayed a short half-life of 5 to 10 min.

Previous research has indicated that p53 can alter normal fibroblasts so that they become tumour-bearing cells[24]. However, later research revealed that dominant-negative mutants were present in a conserved area of p53 in the initially cloned p53 cDNAs utilised in the earlier experiments. Its biological processes and conformation were improved by these changes. This idea was supported by research done in the late 1980s, which also demonstrated that the wild-type p53 functioned as a tumour-suppressing gene and prevented transformation of oncogene[14]. Also, it was demonstrated that the p53

gene commonly had mutations in human large intestine cancer. These results and subsequent research demonstrated that p53 functioned as a tumour-suppressing gene. In reality, numerous investigations have supported the widely accepted theory that authentic wild-type p53 functions as a tumour-suppressing gene and exhibits certain wild-type p53 features. The key points from p53 study in this field could be described in the following way:

First, a mutant p53 gene combined with RAS activation can prevent transformation. Second, the p53 gene mutation is the most frequent gene change found in human cancers. There are regional differences in the p53 mutational profile[25] and disruption of normal p53 activity frequently promotes cancer inhibition and advancement[26]. Finally, mutant p53 proteins might function as suppressive proteins that decrease the action of wild-type p53 proteins by competing with them[27]. Additionally, MDM2-p53 binding can be blocked by modified p53, which kills tumour cells that overexpress MDM2. The combined approach for the destruction of MDM2-p53 connection with radiation revealed improved anticancer effects *in-vitro* and *in-vivo*, as proven by Koom *et al*[28]. As a transcription factor, p53 exhibits tumour-suppressing properties *via* controlling the transcription of genes[14]. p53 has a shallow level of activation in healthy cells. A variety of stress signals, such as DNA damage, oncogene activation and hypoxia, are detected by p53[14]. As a result, in response to stress signals, p53 is overexpressed and accumulates in high quantities. The expression of the relevant genes is then transcriptionally regulated by p53 after it attaches to a particular DNA sequence known as the p53 responsive element (RE) in the DNA. The cellular roles of p53 gene have been identified through studies, including regulation of cell cycle, apoptosis, senescence, DNA repair, alteration of metabolism and metastasis[29,30]. The interplay of certain p53 functions with particular oncoproteins, like MDM2. The following sections of this review go into further detail about these interactions. Posttranscriptional changes primarily regulate the levels of the p53 protein. Ubiquitination, phosphorylation, acetylation, methylation and SUMOylation are a few of these alterations. Ubiquitination is the primary modification that controls p53 in comparison to other changes[31] and a variety of deubiquitinating enzymes control p53 stability[32].

MDM2, a negative regulator of p53, is the primary controller of p53[14]. MDM2 participates in this mechanism by directly interacting with p53 and mediating its ubiquitination-dependent destruction (Figure 2). Fourth, a molecular similarity of MDM2, that attaches to the p53 transcriptional activation region and blocks it from activating transcription, has been discovered in the MDM4 (MDMX) protein [14]. MDM4 thereby aids in the development and operation of tumours. It's essential to note that MDM4 can prevent the breakdown of the MDM2 protein by interacting with the MDM2 protein through the RING finger domain, controlling the function of MDM2 in suppressing p53 activity. As a result, both MDM4 and MDM2 are regularly upregulated in several types of human malignancies. Schlaeger *et al* [33] have demonstrated that MDM4 and EFF1A2 could function as etiology-independent oncogenes in a sizable portion of human HCC.

PATHOGENESIS OF P53-ASSOCIATED HCC

The mechanisms described in this section, which may also apply to other cancer types, have been shown to promote the progression of HCC by changing the MDM2-p53 pathway[34]. As numerous ribosomal proteins (RP) that link to MDM2 are secreted from the nucleolus when ribosome biogenesis is hindered, it has already been demonstrated that p53 stabilized by ribosome biogenesis disruption[35]. There is further proof that a decrease in rRNA synthesis can stabilize p53 by blocking MDM2-mediated p53 damage by binding to RPs released from the nucleolus in the regenerating rat liver following partial hepatectomy[34,36]. It has been established that the p53-binding HBx, which localizes p53 to the cytoplasm, is crucial for the growth of HCC[34]. In cells that possess the HBx protein, doxorubicin therapy has been found to raise p53 levels; in addition, doxorubicin therapy recovers p53-mediated transcriptional function by lowering MDM2 amounts and raising p53 nuclear accumulation[34,37]. Similar to this, the tumour suppressor KLF6 has been found to be inactivated in response to HCV infection, and KLF6 expression has been linked to a poor prognosis for HCC[38]. The Kruppel-like 6 C2H2 zinc finger family, which has been linked to cell differentiation, signal transduction, and cell cycle control, includes KLF6[34]. According to Tarocchi and colleagues, reduced KLF6 expression results in increased MDM2 and decreased p53, and these disparities in the MDM2-p53 pathway is additionally linked to reduced survival in patients with surgically removed HCC[34]. In contrast, increased KLF6 expression decreases MDM2 expression and increases p53 expression in HCC cell lines[39]. In order to boost MDM2 ubiquitin ligase activity and p53 degradation, Jung *et al*[40] discovered the Enigma LIM domain protein, which plays a role in signal transduction *via* protein kinases[34,40].

Serum response factor (SRF), which is likewise elevated in HCC and promotes additional MDM2 stabilization and p53 degradation, can also trigger Enigma[40,41]. By causing MDM2 phosphorylation *via* Akt signalling, Minand and colleagues demonstrated that one p53 target gene, phosphatase of regenerating liver 1 (PRL-1), which is overexpressed in a number of cancers *via* an unknown mechanism, firmly downregulated p53 levels and inhibited p53-mediated apoptosis, forming yet another feedback loop that contributes to HCC development[34]. According to research, Inhibitor of Growth-1 is a type II tumour suppressor that influences cell activity by changing the chromatin's structure and

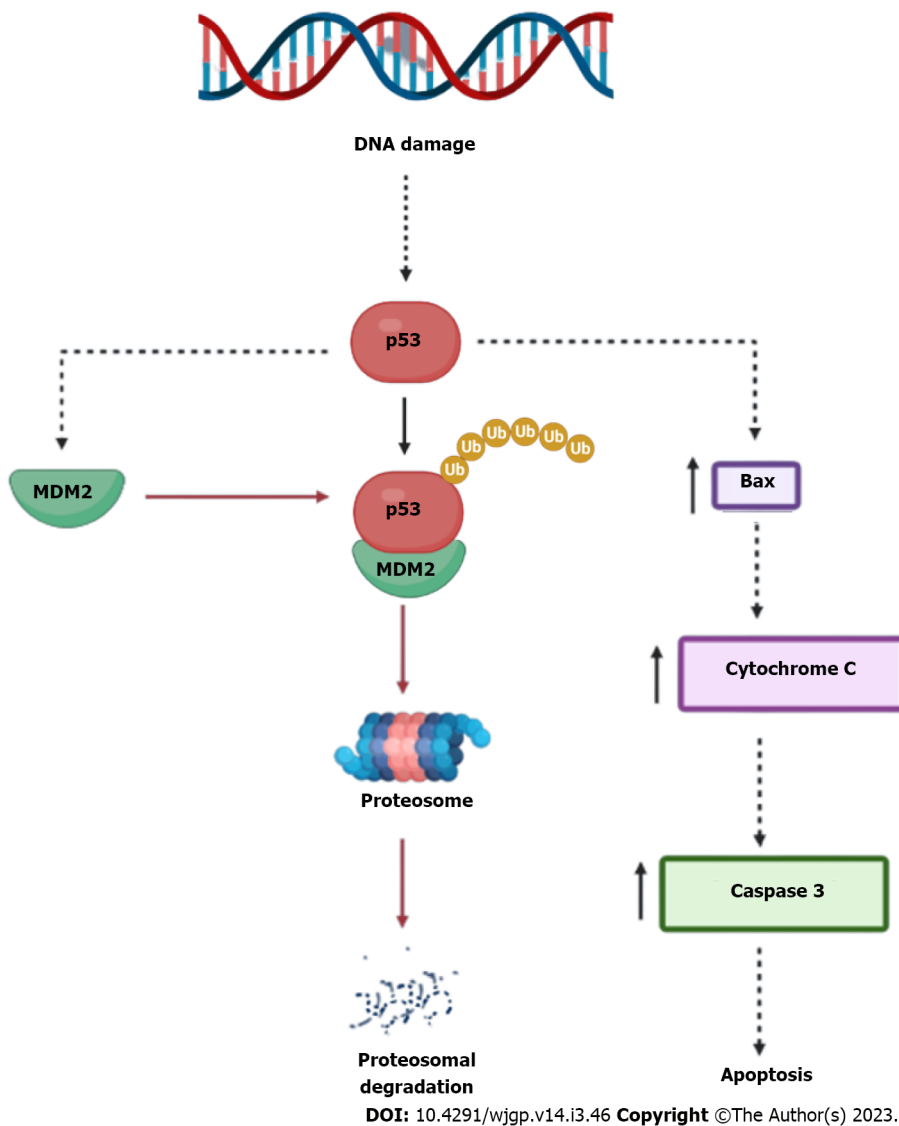


Figure 2 Regulation of p53. p53 is a tumour suppressor protein which leads to increase in the apoptosis markers like Bax, cytochrome C and caspase 3. As murine double minute 2 complexes with p53 it leads to ubiquitination of p53 and proteasomal degradation takes place (Created with BioRender.com). MDM2: Murine double minute 2.

controlling transcription[42]. According to research by Meng *et al*[34], ING1 suppresses the growth of hepatoma cells by causing apoptosis and cell-cycle arrest[34]. Both an increase in p14ARF expression that inhibits MDM2 and an increase in p53 acetylation and activation are likely responsible for these tumour suppressor functions[34]. It has been shown that iron status affects p53 activity by suppressing MDM2 expression and that this reduction in MDM2 expression prevents the development of HCC[43]. Because p53 has been demonstrated to be linked to growth arrest by lowering iron absorption and intracellular iron concentration through interaction with iron-RE-binding proteins, there may be a feedback loop between p53 stimulation and iron concentration[34]. Sirtuin-3 (Sirt3) is a member of the mammalian sirtuin family that is found in the mitochondria and helps to regulate metabolic activity. It is also linked to the Warburg effect, which is the dysregulation of cancer cell metabolism[44,45].

In addition, Sirt3 overexpression reduced HCC cell growth and promoted apoptosis in HepG2 and HuH-7 cell lines by upregulating p53 protein activity, which is found to be downregulated in human HCC tissue[46]. The balance between MDM2 and p53 is thrown off in HCC[34]. Each of the elements demonstrated has a distinct part in promoting the growth of cancer, although defective p53 is invariably identified as the primary cause of HCC[34]. To provide a better understanding of these pathways and other as-yet-unidentified mechanisms that contribute to HCC formation through the modification of p53, further systematic and thorough research is required[34].

ROLE OF P53 IN MECHANISMS OF HEPATOCARCINOGENESIS

Hepatocarcinogenesis is a multi-step process that includes stages of initiation, promotion and advancement. It is characterized by the collection of genetic and epigenetic alterations. Proto-oncogene and tumour suppressor gene genetic changes are highly significant in the development of human cancer, according to molecular genetic studies[47]. In fact, the development of HCC is attributed to over 20 genes linked in 4 carcinogenic pathways[48]. These numerous genetic changes appear to be linked to the development of tumours and multistep carcinogenesis. During chemically generated HCC in rodents, stimulation of oncogenes from Ras family and other families was also discovered; however, it is minimal proof of comparable stimulation in solid cancers[49]; changes in oncogenes have indeed been identified in only a tiny portion of human tumours[50]. Contrarily, there's proof that tumour-suppressing genes, including ones for p53, p16INKa and pRb, are changed at various phases of hepatocarcinogenesis, which may either directly or indirectly lead to chromosomal destabilization and encourage cell growth and neovascularization[51,52]. A variety of human cancer forms, including HCC, have been shown to frequently exhibit mutations in the residual genotype and deletion of one genotype of the p53 tumour-suppressing gene[53,54] (Figure 3).

p53 function

The p53 protein is known to act as a transcriptional factor, however, it may still be discovered to have additional roles[55-57]. It induces the transcription of several genes implicated in the management of the cell cycle, such as the growth arrest and DNA damage-inducible gene GADD45[58], the gene encoding p21WAF1/CIP1 (also known as p21), the gene encoding MDM2 (a negative regulator of p53) and the gene encoding 14-3-3 (a regulator of G2-M progression)[59]. Additionally, it stimulates a number of genes that are likely involved in apoptosis, such as the gene for Bax, a pro-apoptotic protein linked to Bcl-2 and a number of genes that encode proteins included in the production of reactive oxygen species (ROS)[55,57]. The regulation of the cell cycle, DNA synthesis and repair, cell differentiation, transcriptional repression, genomic plasticity and apoptosis have all been linked to p53[53,60]. This includes stopping the cell cycle at G1 and G2 to evaluate genomic integrity. It is crucial for the detection of DNA damage and the subsequent reaction[61]. Cells express more p53 when their DNA is compromised. The cell can then activate the DNA repair systems because this protein prevents the G1 phase. p53 overexpression triggers apoptosis when the damage is too severe to be repaired, which results in cell death[62,63].

p53 regulation

Numerous levels of strict regulation are in place for the p53 protein. Protein activity, protein stability and subcellular distribution are recognized as the three main levels of control[61]. Recently, thorough reviews on additional p53 regulation topics, like p53 post-translational changes and how they affect p53 activities, have been reported[64]. The cell cycle regulates how the p53 protein moves among the cytoplasm and nuclear compartments[65]. For p53 to function as a tumour suppressor, it must build up in the nucleus. An effective way for tumour cells to remain to multiply in the existence of wild-type p53 is to avoid nuclear aggregation[61]. In fact, cytoplasmic sequestration of p53 has frequently been seen in a variety of tumours, including neuroblastomas, breast cancer and colon cancer[66,67]. MDM2 is the factor that causes p53 to accumulate in the cytoplasm of at least some of these tumours and other proteins have also been linked to cytoplasmic retention[68-70]. Many viral proteins, including the human papillomavirus E6 protein[71], the adenoviral E1B 55 kDa protein and the HBV HBx protein[72,73] affect the location of p53. Additionally, several tumour types have indeed been linked to p53 import/export abnormalities[74]. p53 is a protein that shuttles back and forth through the nuclear pore complex. A bipartite nuclear localization signal (NLS) found at the C-terminal domain of p53 and two nuclear export signals found in the N- and C-terminal areas are responsible for nucleocytoplasmic transport. NLS mutations impede MDM2-mediated cytoplasmic breakdown and inhibit p53 export[75].

p53 alterations and hepatocarcinogenesis

A damaged p53 gene, which is typically rendered inactive *via* a single mutation or a gene deletion, is implicated in about 50% of all malignancies. These modifications are thought to hinder p53 from oligomerizing and forming the tetrameric complexes which link to particular DNA sequences, changing the way the wild-type protein behaves physiologically[53,63,76]. Human malignancies with a p53 mutation are more deadly, aggressive and likely to metastasize. Therefore, p53 abnormality detection may reveal details about the molecular pathogenesis and -etiology of human cancer[77,78].

The two most common genetic changes associated with human HCC are mutations of p53 gene and HBV DNA incorporation into the host gene. An abnormality in the p53 gene has been found in 30%-60% of HCC patients[79] and a hotspot of mutations in the gene has been identified in HCC patients from locations with significant aflatoxin exposure[80]. Aflatoxin B1, which contaminates foods in regions where it is endemic, clearly plays a part in the development of hepatocarcinogenesis because its metabolites encourage the formation of apurinic sites and G to T mutations in chromosomal DNA and the presence of a codon 249 G to T transversion in p53 in 50% of HCC patients from high aflatoxin exp-

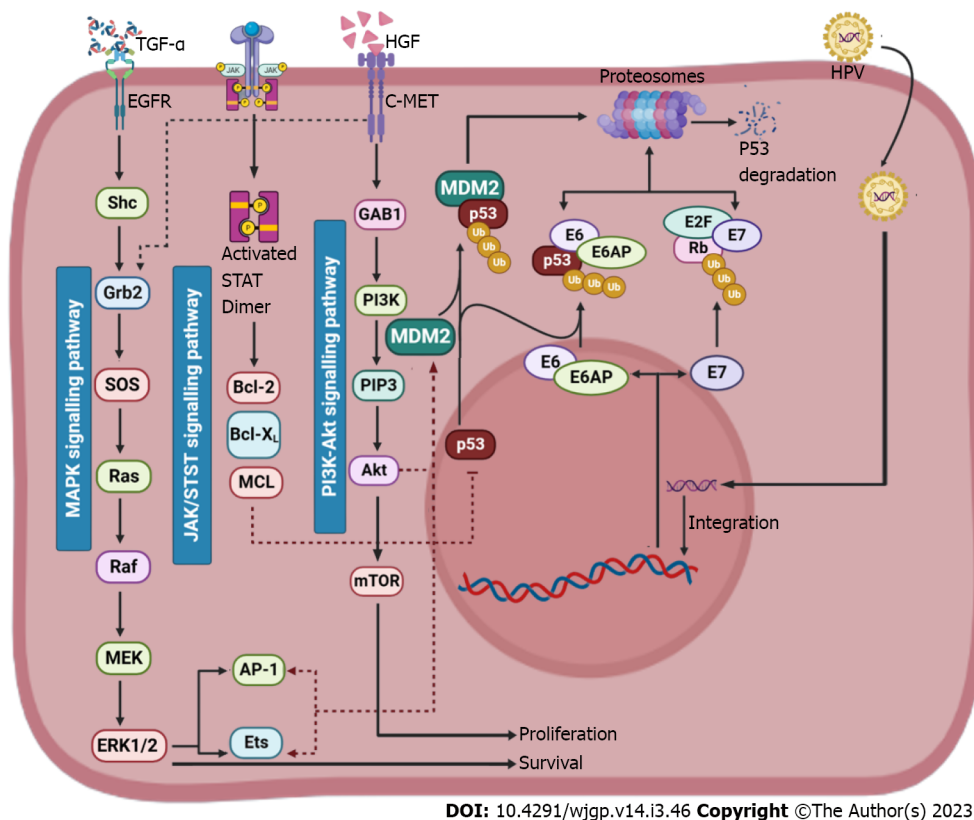


Figure 3 Role of p53 in pathogenesis of hepatocellular carcinoma. Murine double minute 2 (MDM2) suppresses the tumour suppressing activity of p53 by formation of a MDM2-p53 complex. The activation of mitogen-activated protein kinases and PI3K-Akt signalling pathway activates the production of MDM2 and the activation of JAK/STAT signalling pathway suppresses the p53 protein (Created with BioRender.com). TGF: Tumour growth factor; HGF: Hepatocyte growth factor; EGFR: Epidermal growth factor receptor; HPV: Human papillomavirus; MAPK: Mitogen-activated protein kinases; MDM2: Murine double minute 2; MEK: Mitogen-activated extracellular signal-regulated kinases; ERK1/2: Extracellular signal-regulated kinase 1/2; mTOR: Mechanistic target of rapamycin; AP-1: Activating protein-1; MCL: Medial collateral ligament.

osure areas[80].

The transactivation domain of wild-type p53 is polymorphic at residue 72, where a single-base mutation results in the substitution of proline for arginine (CCCCGC)[81]. Numerous research has been conducted on the prevalence of these two alleles and their potential association with the chances of developing cancer, even though the clinical significance of the variants is unknown[61]. According to one study, hepatocarcinogenesis may be influenced by the frequent loss of the proline allele in HCV-associated liver cancer. To clarify this claim, however, further research on this topic has to be done[81]. Furthermore, Okada *et al*[82] discovered a strong association between HCV type 1b infection and male homozygotes for p53Pro. Therefore, there may be a connection between this p53 polymorphism and HCV infection.

ROLES OF P53 IN EXTRINSIC FACTOR-INDUCED LIVER CARCINOGENESIS

Aflatoxin B1

Humans can consume aflatoxin B1 (AFB1), a very known mutagen of the liver made by the *Aspergillus fungus*, through contaminated food products[83,84]. According to one research, the population inhabitants' risk of HCC caused by AFB1 is 17% in some regions of the world[85]. According to the mechanism, CYP40s convert AFB1 into AFB1-8,9-epoxide, which then interacts with DNA to generate 8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B1 (AFB1-N7-guanine) adducts[86]. If these adducts are not repaired, they cause G>T transversions to occur while DNA replication[87,88]. The DNA binding domain of p53 is known to be altered by an arginine to serine missense mutation at codon 249 (R249S), that is brought on by a G>T transversion at codon 249's third base[89]. About 50% of HCC cases in places where AFB1 Levels are high, like Qidong City in China, contain the p53 R249S mutation[90], suggesting that p53 might have a role in AFB1-induced HCC[86].

In addition to the guanines at codon 248, AFB1-8,9-epoxide combine with the p53 gene, although those guanine adducts need not produce cancer-inducing alterations as often as p53 R249S[88,89,91]. While p53 is first activated by AFB1-mediated DNA damage to cause cell cycle arrest during the S to

G2/M phases[92], liver cells which acquire p53 R249S will indeed bypass this cellular defense process with a preferential benefit for proliferation, that might further progress towards liver cancer[86]. In fact, p53R249S is demonstrated to boost insulin-like growth factor 2 (IGF-2) transcription in Hep3B (p53 null) cells, indicating a potential gain-of-function activity[86]. IGF-2 has been linked to accelerating the course of human HCC and is overexpressed in 16%-40% of cases[93]. Additionally, there is a strong association between the genotype IGF-2 +3580 AA and the threat of HCC. It's interesting to note that HepG2 cell viability and proliferation are reduced when IGF-2 is silenced[86]. As a result, p53 mutation caused by AFB1 is critical for the development of HCC, presumably through increased IGF-2 signalling[86].

Vinyl chloride

It is debatable whether vinyl chloride (VC), a carcinogenic gas involved in the production of polyvinyl chloride, can cause human HCC, but it primarily causes angiosarcomas of the liver (ASL)[94-96]. The liver's CYP2E1 enzyme converts VC from the lungs into chloroethylene oxide, which causes liver cancer by forming large DNA adducts[97,98]. 7-(2-oxoethyl)-deoxyguanosine, 3,N4-etheno-deoxycytidine, 1,N6-ethenodeoxyadenosine and N2,3-etheno-deoxyguanosine are four VC-associated DNA adducts that have been identified *in-vivo*[97].

According to reports, the p53 gene's codons 179, 249 and 255 had more A>T transversions in VC-induced human ASLs[99,100]. According to a study employing Sprague Dawley rats, the most of p53 mutations in HCC and ASL after exposure of VC, are A>T transversions; the A>T transversions in ASLs are found at 253 codons of rat p53, that is equal to 255 codons in humans[86,101]. A conformation-specific p53 antibody, PAb240 and other p53 antibodies have been used to identify an increase in the quantities of mutant p53 protein in serum samples from workers exposed to VC[99,102].

Alcohol

Drinking alcohol regularly is bad for our health. It causes liver cirrhosis, which then develops into HCC. One of the major causes of HCC is alcoholic liver disease[103,104]. Alcohol usage is linked to an up to twofold increased risk of HCC, say case studies from throughout the globe[105,106]. Studies on mice on an alcohol diet have also revealed exacerbation of inflammation, epithelial-mesenchymal transition (EMT), fibrosis, as well as progression to HCC[105]. However, hazardous by-products of alcohol catabolism, such as a buildup of acetaldehyde and free radicals, might affect oxidative stress, apoptotic cell death, necrosis, and necroptosis[106]. Pure ethanol cannot directly induce inflammation and liver damage[106]. Elevated inflammatory cytokine release brought on by ongoing inflammatory pathways leads to the creation of ROS[103,106]. HCC growth is aided by ROS-induced DNA damage, hepatocyte genomic susceptibility, and T-lymphocyte suppression[103].

Additionally, alcohol catabolism affects a number of lipid metabolism processes, which results in liver steatosis and fatty acid oxidation inhibition[105,106].

An essential biological process known as epigenetic reversibility of gene expression frequently contributes to cancer[106]. Alcohol abuse alters epigenetic pathways that result in altered DNA methylation and acetylation. For instance, aberrant acetylation has been linked to alcohol-induced HCC and hepatic steatosis[106]. In a syngeneic BALB/c mouse tumour model, promoter hypomethylation in circulating tumour cells of HCC is strongly correlated with overexpression of c-Met and hepatocyte growth factor[107].

Alcohol consumption also affects metabolism, causing acetaldehyde to accumulate, hypomethylation, an absence of antioxidants and retinoic acid, as well as inflammation, oxidative stress, hypoxia, and genetic instability[106].

Non-alcoholic fatty liver disease

NASH, non-alcoholic fatty liver disease (NAFLD), fibrosis, cirrhosis and HCC are among the illnesses that make up NAFLD. Obesity, type 2 diabetes and metabolic syndrome are all linked to NAFLD[108]. NAFLD affects 20%-30% of people in the West, according to estimates[109]. Just 11.5% of individuals with NAFLD-caused cirrhosis go on to grow HCC and roughly 50% of NASH-caused HCCs happen in people who don't have cirrhosis[110,111]. These findings suggest that additional oncogenic events are necessary for NAFLD-caused HCC[86]. The cellular pathways in NAFLD caused HCC, however, remain poorly known. Its development has been linked to a number of mediators, including insulin resistance, deregulation of NF- κ B signalling, the PI3K/ATK-PTEN pathway and the activity of certain miRNAs (*e.g.*, miR-34)[112,113].

Additionally, p53 has been linked to the development of NAFLD *via* a variety of pathways. In a mouse model for NAFLD, in which p53 +/+ and p53 -/- mice have nourished a diet deficient in choline and methionine, p53 +/+ mice exhibit enhancement in histologically visible steatohepatitis, fibrosis and ROS formation as well as higher protein levels of p66Shc, a protein linked to oxidative stress, in comparison to p53 Human NASH hepatocytes have increased p53 activity along with elevated p21 and p66Shc mRNA levels, that is highly linked to the degree of fibrosis[86]. Additionally, a rise in miR-34 expression and reduced SIRT1 protein content were found in human NAFLD liver tissues along with a rise in acetylation of p53, both of that were associated with the condition[114]. By promoting hepatocyte death, stimulation of the miR-34a-SIRT1-p53 axis has also been demonstrated to lead to liver fibrosis or

NASH[115]. Additionally, p53 has the ability to activate miR-34, which suppresses the expression of SIRT1 mRNA and raises p53's acetylation in a positive feedback loop[116]. These findings suggest that NAFLD and high expression of miR-34 and p53 are related. However, it should be highlighted that p53-dependent and p53-independent apoptosis can be mediated by miR-34a[117]. Nevertheless, some evidence points to p53's involvement in the development of NAFLD and NASH; however, more research is needed to show if p53 actually have a critical role in the development of NAFLD-mediated HCC[86].

Iron overload

Iron is a crucial mineral that is involved in several metabolic processes, including heme production, the formation of the Fe-S cluster and the transportation of oxygen by haemoglobin[118].

Nevertheless, there can be serious implications when iron homeostasis is disturbed, whether as a result of hereditary or environmental factors, such as hepatic fibrosis, cardiomyopathy and arthropathy, endocrine abnormalities[119,120]. Crucially, having too much iron raises the risk of developing a variety of neoplasias, such as breast cancer, colorectal cancer and HCC[121]. Dietary iron excess, primarily in beer cooked in iron pots, is closely linked to a higher risk of HCC in several areas of sub-Saharan Africa [122]. HCC has been demonstrated experimentally in Wistar rats on iron-rich diet[86,123]. Multiple rodent models and investigations of human HCCs have connected higher iron content with increment in 8-oxo-2-deoxyguanosine adducts and oxidizing agents like malondialdehyde, suggesting that ROS-inducing DNA alterations are one pathway involved in iron overload-linked HCC genesis[86,124].

Moreover, there is proof that an excess of iron directly affects p53 activity[86]. A high-iron diet causes p53 protein levels in the liver of C57BL/6 mice to drop[125]. Additionally, male Sprague-Dawley rats exhibit a rise in MDM2 and a reduction in p53 in the liver when fed a high-iron diet over an extended length of time[126]. Another chemical mechanism for why there are fewer p53 molecules in the blood when there is too much iron is that p53 is linked to heme, transferred to the cytoplasm and then destroyed by the proteasomal pathway in HepG2 cells[125]. As a result, p53 levels are decreased by both iron overload and dysregulated heme, which aids in the development of HCC[86]. It's interesting to note that p53 also affects intracellular iron content *via* transactivating iron-sulfur cluster enzyme 2, which results in less efficient iron uptake[127,128]. As a result of chronic iron overload, decreased p53 function raises intracellular iron levels, which further encourages the development of HCC. It is important to emphasized that people with hereditary hemochromatosis have greater frequencies of p53 mutations (64%-71%) than those with sporadic HCC, indicating a possible involvement of p53 in the development of HCC brought on by iron overload[129,130]. 45% A>C transitions and 33% G>C transversions, involving two hotspots at codons 275 and 298, are seen in HCC tissues from hereditary hemochromatosis[86,130]. The p53 mutation spectrum in the research utilizing British families with hereditary hemochromatosis is made up of 60% A>G transitions and 40% A>T transversions, though [129]. However, it is still unclear if iron excess does in fact cause HCC in animal models in a p53-dependent manner[86].

HBV

According to estimates, 248 million people worldwide have chronic HBV infection and are hepatitis surface antigen positive[131]. HBV is the main contributor of HCC, with chronic HBV infection accounting for the majority of cases[86,132]. Repeated episodes of immune-linked hepatocyte death and consequent tissue regeneration can result in cirrhosis of the liver and HBV-mediated HCC carcinogenesis[133]. Importantly, 10%-30% of HCCs linked to HBV do not develop in the context of cirrhosis, pointing to additional oncogenic pathways involved in the development of HCCs linked to HBV[86,134].

The genome of HBV, a circular, partly double-stranded DNA virus, has four overlapping open reading frames: A core area, a surface region, a polymerase region and an X region[86]. These four regions together develop seven viral proteins: Precore, polymerase, S, M, core, HBx and L[135,136]. The HBx protein, which is crucial for viral replication and is most connected to the development of HCC, is one of these[136]. In fact, HBx causes HCC in transgenic mice models by confining p53 to the cytoplasm [137,138].

Additionally connected to hepatocyte apoptosis is HBx[86]. In many situations, HBx prevents apoptosis by adhering to and sequestering p53 in the cytoplasm in addition to raising levels of the anti-apoptotic protein surviving[69,139]. Additionally, HBx is said to suppress tumour growth factor (TGF)-mediated apoptosis[140]. On the other hand, in rare circumstances, HBx has been demonstrated to cause apoptosis without the involvement of p53[141,142]. The double functions of HBx in hepatocyte apoptosis and its connection to the development of HCC, therefore, call for additional research[86].

Although there is no concrete proof for it, HBx variants with C-terminal truncations (Ct-HBx) are typically found in HCC and may potentially have a role in the progression of HCC[143,144]. In numerous cell lines, Ct-HBx suppresses apoptosis and encourages hepatocyte growth[145]. It has also been demonstrated that Ct-transcriptional HBx's repression of USP16 (ubiquitin-specific peptidase 16) enhances the tumorigenicity and stem-like characteristics of HCC cells[86]. Additionally, like HBx, Ct-HBx links to p53 and blocks p53-linked apoptosis[135]. Likewise, few Ct-HBx variations have the capacity to inhibit GAS2, a modulator of p53-linked apoptosis, from expressing its mRNA[146]. As a

result, USP16 is downregulated and p53-mediated apoptosis is inhibited, which may help explain how Ct-HBx contributes to the pathogenesis of HBV-related HCC[86]. Given that p53 mutations are uncommon in HBV-related HCC, are linked to advanced disease and are inhibited by both HBx and Ct-HBx, suppression of p53 activity might be more advantageous for HBV-linked HCC carcinogenesis than p53 mutation[147]. Crucially, HCC patients with wild-type p53 outlive those with p53 mutations in terms of both overall survival and recurrence-free survival[86].

HCV

According to estimates, 184 million people worldwide have anti-HCV positivity and those who have HCV have a 15-to-20-fold higher risk of developing HCC[148]. HCV is a single open reading, positive sense, 9600 nucleotide long single-stranded RNA virus[149]. The HCV genome produces a polyprotein that is broken into nine viral proteins, encompassing structural proteins (C, E1 and E2) and non-structural proteins (N, S and S2) (p7, NS2, NS3, NS4A, NS5A, NS5B)[86,150]. Although cirrhosis is the primary setting for the great majority of HCCs connected to HCV, there is some proof that the HCV viral proteins have the capacity to cause cancer[134]. Particularly, both p53-dependent and -independent mechanisms have been proposed to link the development of HCC to the HCV core, NS3 and NS5 proteins[86].

Without the presence of cirrhosis, HCC definitely instantaneously arise in transgenic mice that produce the HCV core protein[151]. The Wnt- β -catenin pathway is upregulated, ROS levels are increased and Fas- and tumor necrosis factor (TNF)-mediated apoptosis is inhibited by HCV core protein[86]. The core protein alters the subcellular localization of p53 to the perinuclear area and nuclear granular structures, as well as its post-translational changes, such as phosphorylation and acetylation, in HeLa and HepG2 cell lines[86]. This is significant because it reduces p53 activity[152]. Additionally, the core protein activates SIRT1, which deacetylates p53, impairing p53-dependent apoptosis in HepG2 cells. Thus, both p53-dependent and -independent mechanisms by which HCC is caused by HCV core protein are likely[86].

Another HCV protein that could alter human hepatocytes is called NS3, a non-structural HCV protein that increases cyclooxygenase-2 and activates mitogen-stimulated protein kinase[153]. In HeLa and NIH3T3 cells, NS3 also forms complexes with p53[154] and inhibits p53's transcriptional activity in NIH3T3 and Huh7 cells. Furthermore, overexpressing NS3 can cause NIH3T3 cells to convert and cause tumours in animals[155]. It is yet unknown, nevertheless, whether or not p53 is required for NS3-mediated transformation. In transgenic mice models, the non-structural HCV protein NS5A can result in steatosis and HCC[86]. It has been demonstrated that NS5A inhibits Bax-linked apoptosis, transactivates c-fos and prevents TNF-linked apoptosis without the need for p53[156]. However, p53's transcriptional activity can be inhibited when NS5A binds to and colocalizes with p53 in the perinuclear membrane. Additionally, NS5A interacts with hTAFII32 at the nucleoplasm membrane and prevents it from stabilizing p53, which prevents p53-mediated apoptosis in Hep3B cells[157]. As a result, NS5A promotes the growth and progression of HCC through both p53-dependent and -independent mechanisms[86].

Relationship between HCV Core protein and p53 in HCC

The structural viral protein known as the HCV core protein encases the viral genomic RNA. According to its subcellular localization and the subsequent impact on the cell cycle inhibitor p21, the core protein may also have contradictory effects on cell growth, supporting both apoptosis and cell proliferation[158, 159].

The innate version of the HCV core protein is synthesized first (amino acids 1–191) and a mature form (amino acids 1–173) is created during processing[61]. The innate form and the mature form, form a heteromultimer in the cytoplasm, preventing the mature form from being transported to the nucleus. The innate form activates p53 in the cytoplasm and p53 then increases the production of p21 by acting as a transcription factor for p21. It is unclear how the innate form stimulates p53, but because it binds to the protein, it may do so by stabilizing the protein[159,160]. p21 expression can be decreased by a process unrelated to p53 if the amount of mature core protein penetrates the nucleus and surpasses the innate form's binding capacity[161,162]. A core-responsive region in the p21 promoter precisely overlaps a butyrate/TGF-RE. In this instance, core protein stimulates a butyrate route to activate p21 *via* the element[73].

Additionally, the HCV core protein controls p73, a tumour suppressor protein belonging to the p53 family[61]. In addition to being engaged in neurogenesis and innate immune responses, p73 also appears to have a significant role in the development and maintenance of malignancies[163]. The HCV core protein is translocated into the nucleus as a result of its interaction with p73. Additionally, in a p53-dependent way, the association with the core protein blocks p73- α , but not p73- β , dependent cell growth arrest. Consequently, the impact of the HCV core protein on p73 function may play a role in HCV pathogenesis[61].

Relationship between HCV NS3 protein and p53 in HCC

The HCV NS3 protein may cause host cells to become hepatocarcinomas at an early stage. Additionally, it has been suggested that it might result in p53 gene mutation and hepatocyte transformation, however

this is debatable[164,165]. NS3 and wild-type p53 combine to form a complex[164]. The oligomerization domain, located close to the C-terminus of wild-type p53[166] (amino acids 301-360), is crucial for the formation of this combination with NS3. In line with this discovery, NS3 protein can specifically and dose-dependently suppress the promoter activity of p21 *via* altering the activity of p53[61]. The impact works in concert with that of the HCV core protein and is not cell-type specific.

Relationship between HCV NS5A protein and p53 in HCC

A phosphoprotein between 56 and 58 kDa, the HCV NS5A protein. Although NS5A is a member of the viral replicase complex, which is located on the cytoplasmic side of the endoplasmic reticulum, along with other virus-encoded proteins, its function in viral replication is yet unknown[61]. Phosphorylation and possible proteolytic processing of NS5A into lower molecular weight forms that can translocate to the nucleus are examples of post-translational changes[167]. Truncated forms of NS5A can act as transcriptional activators, but other now has discovered interrelations of NS5A with cellular proteins, such as those of the transcription activation machinery, cellular signalling apparatus and cell-cycle-regulatory kinases, suggesting that it plays a pleiotropic role in HCV-host interrelations[167,168]. Numerous of these interactions prevent the apoptotic cellular response to persistent HCV infection, that raises the possibility that NS5A plays a role in the development of chronic liver conditions and HCC linked to HCV infection[61].

By locating p53 in the perinuclear area, NS5A can prevent it from binding to certain DNA sequences. Inhibiting p21 expression and p21-mediated apoptosis in this manner[169]. Additionally, p53 and TATA-box-binding protein (TBP) connect to NS5A, generating a heteromeric complex that prevents p53 and TBP from attaching to their respective DNA binding regions. In addition, p53-TBP-excision repair cross-complementing factor 3 complex formation is inhibited by NS5A[61]. Additionally, the NS5A protein may prevent p53 from activating by securing the crucial coactivators of p53, hTAF(II)32 and hTAF(II)28. Recently, it was discovered that the protein kinase PKR, which is activated by interferon (IFN), is inhibited by NS5A. Thus, NS5A may be involved in HCC's IFN resistance[169,170].

OTHER MECHANISMS OF PROGRESSION TO CIRRHOSIS AND HCC

Other potential risk factors for the development of HCC include genetic predisposition, prenatal anomalies, chemical exposures (aflatoxin or food tainted with arsenic), and autoimmune illnesses of the body's liver[106].

Patients with a number of congenital defects are more likely to develop liver cirrhosis and HCC. These consist of hemochromatosis, alpha-1-antitrypsin deficiency, Wilson's disease, and hereditary tyrosinemia[171]. AFB1-induced HCC is caused by a number of processes, some of which involve the development of mutagenic and carcinogenic intermediates and adducts. Food contaminated by the fungus *Aspergillus flavus* and *Aspergillus parasiticus* releases aflatoxins. There are several chemical changes that happen as a result[106].

AFB1 is transformed into the following known mutagenic or carcinogenic compounds: 8,9-dihydroxy-8-(N7), aflatoxin-B1, aflatoxin B1-8,9 exo-epoxide. Aflatoxin B1 form aminopyrimidine adduct guanyl-9-hydroxy aflatoxin B1 adduct[106]. Additionally, these intermediates and adducts have the ability to directly cause a mutation at codon 249 of the p53 tumour suppressor gene[106]. This changes the amino acid arginine to serine, reversing the gene's ability to prevent tumour growth[106]. According to certain reports, AFB1 and HBV may work together to cause HCC[172]. Additionally, additive interactions have been documented. In a systematic review, Ogunwobi *et al*[106] showed a connection between autoimmune hepatitis (AIH) and an elevated risk of developing HCC in individuals with liver cirrhosis. The chance of developing liver cirrhosis from AIH was determined to be less than the probability of developing the condition as a result of HBV, HCV, or primary biliary cholangitis. But the risk of HCC and liver cirrhosis from AIH is clinically considerable[106].

MDM2-P53 INTERACTIONS IN HCC

It has been demonstrated that MDM2 inhibits p53 gene transcription and translation in addition to adversely controlling p53 functions through ubiquitination and degradation[173]. The cell fate as well as the balance between MDM2 and p53 may be impacted by factors that control the ubiquitination of MDM2 and p53[174]. The connections between MDM2 and p53 are regulated by various mechanisms [14,175]. Ones of these interactions could be.

RPs

RPs have a substantial impact on nuclear strain, cell cycle arrest and apoptosis *via* activating the MDM2-p53 regulatory loop[176]. Knockdown of nuclear proteins can cause nuclear/ribosomal stress by sequestering MDM2 and inhibiting its ubiquitin ligase function against p53[177], which in turn causes

nuclear/ribosomal stress[35]. It has been demonstrated that ribosome biogenesis interruption leads to p53 stability. This is caused by a number of RPs that are linked to MDM2 and restrict its action for p53 being released from the nucleolus[35]. As a result of blocking E3 ubiquitin ligase, the subunit RPs rpL5, rpL23 and rpL11 have demonstrated the capacity to attach to MDM2. According to Zhang *et al*[178], the human protein HDM2, which is the MDM2 ortholog, interacts to RP L11[14]. The docking site is located in the middle of HDM2, which prevents HDM2-associated p53 ubiquitination and destruction and restores p53-linked transactivation and p21 protein deposition. The research confirmed the idea that L11 controls HDM2 negatively[14].

Ribosomal RNA

The most prevalent element found in ribosomes is ribosomal RNA, which is the RNA portion of the ribosome and is necessary for protein synthesis[14]. In human tumour cells, it has been discovered that RNA polymerase I subunit A (POLR1A) gene silencing results in a decrease of rRNA production that stabilizes p53 without altering the integrity of the nucleolus. The suppression of the MDM2-mediated p53 breakdown was what caused p53 to stabilize. However, rRNA production down-regulation that was accompanied by a decrease in protein synthesis did not result in the stability of p53[14]. The results showed that p53 functions in mammalian cells are controlled by the equilibrium of RP and rRNA production[36].

MicroRNAs

Small non-coding RNAs called microRNAs (miRs) regulate the transcription and post-transcriptional expression of many target genes. In cancer, their action is typically dysregulated[14]. Micro-RNAs generally decrease in human malignancies, suggesting that they could be involved in tumour suppression. Therefore, by focusing on oncogenes or tumour-suppressor genes, miRs can perform a variety of biological roles[14]. The first miR-34 family members to be implicated in the p53 tumour-suppressing network was identified in 2007 and are known as miR-34a, miR-34c and miR-34b[179]. The expression of members of the microRNA family (miR-34a-c) evidenced the p53 condition and the genes encoding miR-34 family members were transcriptional domains of p53 *in-vivo* and *in-vitro*, according to the authors' comparison of the microRNA expression profiles of cells with and without p-53 deficiency [14]. As a result, the p53 network inhibited the development of tumours *via* a variety of co-ordinated interactions and multiple transcriptional targets, such as the function of the miR-34 family members in preventing uncontrolled cell growth[14].

Bi *et al*[180] investigated the function of miR-125a in HCC. MiR-125a expression was found to be downregulated in HCC tissues and cell lines and it was linked to aggressive pathological characteristics [14]. According to the research, miR-125a targets vascular endothelial growth factor A and matrix metalloproteinase II, which both function *in-vivo* and *in-vitro* to prevent the progression and metastasis of HCC. As a result, miR-125a overexpression might be an effective treatment for HCC.

Experiments on miR-125b's function revealed that it negatively regulated p53 and p53-caused apoptosis in cells under stress, implying that it had a tumourigenic effect, for example[181]. miR-122, one of the several miRs found in liver tissue, has been demonstrated to function as a tumour suppressor in HCC[14]. Poorly differentiated cancer, big tumours, metastasis and a poor prognosis are usually linked to decreased miR-122 expression[182]. Simerzin and colleagues recently demonstrated that miR-122*, the passenger strand of miR-122, targeted MDM2 and had an active role in the association between MDM2 and p53. The levels of miR-122* and MDM2 were significantly inversely correlated in human HCC samples. By highlighting the tumour-suppressor properties of miR, *in-vivo* research demonstrated that miR-122* was capable of preventing the formation of tumours[14]. Inhibiting miR-122 using an antagomiR-122 (miR inhibitor) caused miR-122* to build up, MDM2 to be suppressed and the p53 protein to be increased. The research revealed that miR-122*, the miR-122 passenger strand, controlled the function of p53 by concentrating on MDM2. As a result, miR-122* exhibits tumour-suppressive action that was previously only associated with miR-122. Additional research is required to determine whether it is useful as a treatment for HCC as well as what key distinctions occur among miR-122* and miR-122[14].

Kim *et al*[183] discovered that p53 increased the expression of members of the miR-200 and miR-192 families and explained the function of p53 in controlling the EMT in HCC *via* the development of particular effector miRs, such as miR-141, miR-192, miR-194, miR-200b, miR-200c and miR-215[14]. These miRs specifically targeted the zinc-finger E-box-binding homebox 1 and 2 (ZEB1 and ZEB2) and EMT-activity transcription factor 2. *ZIB1* and *ZEB2* genes were repressed in human HCC and HCC cell lines by p53 overexpressed miRs. This reduced EMT resulted in enhanced E-cadherin and reduced vimentin expression[14].

LIM Domain protein Enigma

It has been demonstrated that the protein Enigma, PDLIM 7 (Enigma LIM), which has a LIM domain, interacts with proteins included in protein kinases and that the Enigma gene has been detected in stomach and liver malignancies in humans[14]. It's also been demonstrated that even *in-vitro*, in human hepatoma cell lines and in mice embryonic fibroblasts, the LIM domain protein Enigma actively links

with MDM2 to create a complex with p53[14]. Furthermore, it has been demonstrated that the Enigma caused p53 breakdown by reducing MDM2's ability to ubiquitinate itself and boosting its ability to bind to p53[40].

SRF

Serum growth factors were capable of rapidly stimulating the expression of HDM2 in a well-studied non-transformed human fibroblast model, as demonstrated by Phillips *et al*[184]. The authors noted that the increased HDM2 protein, that also accompanied growth factor activation, was mainly the consequence of phosphatidylinositol-3 kinase-dependent stabilization of the HDM2 protein along with a global rise in protein synthesis in their investigation, which investigated the mechanistic reaction for these variations[14]. According to a new study, SRF induction enhanced Enigma transcription, stabilizing HDM2, which then caused p53 to degrade[14]. The authors saw these modifications in the liver given the hepatocyte growth factor. Additionally, they discovered that MDM2 co-expressed with SRF and Enigma, but not p53, in the human stomach and liver tumours. Enigma was also discovered to decrease p53-linked apoptosis in cell lines and a mouse xenograft model, which boosted cell viability and produced chemoresistance[14].

PRL-1

Several malignancies, including colorectal[185], gastric and liver tumours, and especially metastatic cancers, have been discovered to overexpress the protein tyrosine phosphatase family of the PRL family. PRL-1, PRL-2 and PRL-3 displayed a higher level of structural sequence similarity within this family. According to Min *et al*[186], overexpression of PRL-1 prevented p-53-mediated apoptosis and decreased levels of endogenous and exogenous p53[14]. The ubiquitination of p53 and subsequent proteasomal degradation were the mechanisms by which p53 was down-regulated. The stimulation of PIRH2 (p53-induced RING-H2) transcription and the promotion of MDM2 phosphorylation *via* Akt signalling were the two main strategies used to ubiquitinate PRL-1[14]. These results suggested that the growth of cancer was aided by the down-regulation of p53 caused by a PRL-1-induced negative feedback mechanism. Additional research by the same team showed that PRL-3 activated PIRH2 and MDM2 in cancer cells, negatively regulating p53 in a manner akin to PRL-1[187].

MDM2 binding protein

The MDM2 binding protein (MTBP) was demonstrated to have metastasis-suppressing properties in earlier investigations. Additionally, it has also been proven that MTBP prevented infiltration irrespective of p53 status[172]. Currently, Bi *et al*[188] established the growth inhibitory action of MTBP in HCC and observed that, when compared to nearby non-tumour tissue, MTBP expression was reduced in almost 70% of human HCC cells[14]. The local invasion of lymph node metastases was also linked to this decrease. Regardless of the p53 status, the transcription rates of MTBP were found to be adversely linked with the ability of HCC cells to migrate[14]. These findings supported the findings of Vlatković *et al*[189], who showed that low MTBP was linked to a poor prognosis for head and neck malignancies. Moreover, there are currently a number of holes in this field. For instance, it is yet unclear how HCC cells control the expression of MTBP. Additionally, it is unclear how functionally related MTBP and MDM2 are. We have to understand why p53 is not required for these modifications in the interim[14]. The existence of a single nucleotide polymorphism (SNP309) in the MDM2 promoter is another element that might influence how p53 interacts with MDM2. It has been demonstrated that this factor raises the affinity of the transcriptional activator Sp1, leading to greater levels of MDM2 RNA and protein as well as a resulting attenuation of the p53 pathway[14]. The authors' suggestion that SNP309 serves as a rate-limiting event in carcinogenesis stems from studies showing that such mechanisms enhance the development of tumours in humans[190].

MECHANISMS OF P53 THERAPY FOR HCC

HCC typically harbours p53 mutations[191]. Despite carrying wt-p53, the majority of HCCs have abnormalities in the p53-mediated apoptotic pathway[192]. In HCC, p53 might occasionally be wild-type but inactive[193]. The expression of wt-p53 can be induced by signals to and activation of p53, which suppresses the altered phenotype of hepatocytes and boosts the actions of radiation therapy and chemotherapy[194]. In HCC, p53 can be partially or completely repressed and single tiny compounds, like the well-known antimalarial medication quinacrine, can activate p53[195]. Exogenous wt-p53 can be delivered into cancer cells by gene delivery, which effectively slows the growth of tumours that contain both mutant and wild-type p53[196]. The oncogene MDM2 generates a phosphoprotein that builds a strong complex with mutant as well as wild-type p53 protein, blocking p53-mediated transactivation and causing p53 breakdown[197]. MDM2 also has a p53-DNA binding site[195]. The MDM2-p53 autoregulatory feedback loop[198] controls the transcription of the MDM2 gene and the activity of the p53 protein. The amount of wild-type p53 protein can control the expression of the MDM2 gene and the MDM2 protein can interact with p53 to reduce its capacity to function as a favorable transcription factor

at the MDM2 gene-responsive region[195]. To boost functional p53 levels and p53-mediated therapeutic efficacy, several methods have now been used to disrupt MDM2-p53 interaction[199]. The protein-secreting pathway is significantly regulated by Adenosine diphosphate (ADP)-ribosylation factor (ARF) proteins[200]. ADP and adenosine triphosphate are converted to adenosine in a human HCC cell line and treating HCC cells with adenosine can decrease their development and activate caspase-3, showing that adenosine is a cytotoxic apoptotic agent[195]. ARF, which is regarded as a tumour suppressor[201], can start the cellular response to abnormal oncogene stimulation by attaching to and preventing MDM2 from functioning[195]. Like the murine protein, the human protein p53's capacity for transactivation can be inhibited by HDM2, which is MDM2's human counterpart[202]. Different locations on MDM2 are where ARF and p53 bind. According to MDM2's mediation, the ARF-p53 complex is created, which can improve p53 stability[203]. Successfully inducing p53-triggered tumour cell death can be achieved by blocking the routes for p53 breakdown, either through over-expression of ARF or by disrupting the connection between MDM2 and p53[195].

High levels of exogenous p53 protein might have therapeutic benefits for HCC in two ways: (1) By increasing tumour cell apoptosis by preventing cell proliferation *via* a number of physiological mechanisms and[123] by making HCC more susceptible to certain chemotherapeutic drugs[195]; and (2) Additionally, due to the wt-p53 protein's angiogenesis blocking properties, p53 reduces the expression of genes related to angiogenesis[204].

Due to medication resistance, treating HCC is extremely challenging. Reversing multiple drug resistance in human HCC cells and figuring out the underlying processes are both important[195]. In HCC cells expressing mt-p53, P-glycoprotein expression and function are increased, as are the products of numerous drug resistance genes (MDR)[205]. Expression of mt-p53 decreases the absorption of chemotherapeutic drugs by HCC cells and increases their treatment resistance. MDR transcription is inhibited by wt-p53 as opposed to mt-p53, which increases MDR expression[206]. Due to the reduced expression of P-glycoprotein encoded by MDR, HCC cells become more sensitive to chemotherapeutic drugs when wt-p53 function is restored in these cells[195].

THERAPEUTICS PRODUCTS WITH P53 STATUS AS A TARGET

Currently, the most frequently used *in-vitro* and *in-vivo* therapeutic agents for the treatment of HCC are the oncolytic virus and recombinant adenovirus p53 (rAd-p53)[195]. Both the expression of p53 and p21 proteins is observed to be up-regulated in cells following the introduction of exogenous wt-p53 generated by the recombinant adenoviral vector. It is possible to stop cell division and apoptosis. Oncolytic viruses are a group of flawed viruses[207] that cannot reproduce in healthy cells but can expand in tumour cells, ultimately lysing them[195].

Recombinant adenovirus p53

Human adenovirus vector processes are typically utilized for the expression of proteins in humans or other species with a few benefits due to their wider host range and lesser pathogenicity to humans, have a binding attraction for epithelium[208], which is essential as the majority of human tumours are of epithelial origin and serotype 4 of species E[209] have a specific binding affinity for HCC cells[208]. It has been determined that the E1 region of the adenovirus, a subregion of the viral genome seen in converted cells, is what causes transformation[195]. Exogenous DNA is used to substitute the E1 region in the construction of recombinant human adenoviruses, which results in replication defects and a negligibly high level of acute toxicity[210]. Recombinant adenoviral vectors that express wt-p53 (Ad-p53) allow for the transfection of the p53 gene into HCC cell lines[195]. *In-vivo* tumour growth of neighbouring non-transduced cells can be inhibited by tumour cells transduced with the wt-p53 gene, according to experiments. This suggests that Ad-p53 is also anti-angiogenic[195,211], at least in part because of the bystander effect caused by the transfer of the wt-p53 gene on neighbouring tumour cells.

Oncolytic viruses

Oncolytic viruses that interfere with the key surveillance pathways, as those mediated by p53, might multiply selectively in them and cause lysis since HCC cells with p53 abnormalities have abandoned their cellular surveillance systems[195]. Adenoviruses have E1A and E1B genes, with E1A being an apoptosis-inducing gene and E1B being an apoptosis-inhibiting gene[195]. The p53 gene is bound to and rendered inactive by the 55kDa protein from the E1B-region of the adenovirus[212]. The 55-kDa E1B protein is not produced due to a loss in E1B and the mutant adenovirus known as ONYX-015[213] can only multiply in cells lacking wt-p53. The E1B55K-defective adenovirus ONYX-015 is a prototype for oncolytic viruses that may specifically reproduce in and kill p53-deficient HCC cells[195]. Unless cancer gene therapy is properly planned based on the biology of a particular tumour type[214], it is unlikely to be successful. Another E1B 55kDa-deficient adenovirus with a mouse endostatin gene has been developed for antitumour activities against HCC and is known as CNHK200-mE to increase the effectiveness of such oncolytic viruses[195]. A novel strategy known as a gene-viral vector or gene-viral therapeutic system has been developed using the synergic activity of a carrier virus and therapeutic

gene[195]. In this method, an anti-tumour gene is inserted into the genome of a replicative virus specific for tumour cells in order to merge the benefits of gene and virus therapies. CNHK300-murine endostatin (CNHK300-mE) is created using the human telomerase reverse transcriptase promoter to activate the production of the adenovirus E1A gene and clone the therapeutic gene mouse endostatin into the adenovirus genome, indicating promising results in the treatment of HCC[195] (Table 1).

P53 GENE IN CHEMORESISTANCE IN HCC

Through the direct inhibition of apoptotic pathways, the buildup of mutant p53 with gain of function can cause tumours to become resistant to treatment[215]. p53 has been linked to chemotherapeutic treatment resistance in HCC. Cisplatin, also known as cis-diamminedichloroplatin (CDDP), is an essential part of standard treatment plans for tumours of the gastrointestinal, pulmonary and genitourinary systems[216]. The response rate to CDDP in systemic monotherapy for HCC is 15%[217], however multidrug regimens containing this medication produce higher response rates[218]. Because arterial infusion schemes with CDDP reach larger concentrations of the drug inside the tumour and consequently exhibit a more potent anticancer effect, response rates range from 41% to 61%[219]. In most HCC cell lines, regardless of p53 status, a prior study revealed that CDDP has a synergistic effect with tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis[218]. But an in-depth examination of the Bel7402 (p53 wild type), Huh 7 (p53 mutant) and Hep3B (p53 deficient) cell lines revealed that the p53 status is only partially necessary for CDDP to reverse TRAIL resistance in HCC cells[220]. Thus, p53 is linked to the effectiveness of CDDP in inducing apoptosis. Later research revealed that defective p53 contributes to CDDP resistance[218]. In HepG2 cells, the long interspersed nuclear element-1 ORF-1 protein (LINE-1 ORF-1p) enhances CDDP and epirubicin resistance by suppressing the production of the p53 gene[221]. As a result, HCC cells are more likely to be CDDP resistant when p53 expression is downregulated[218].

The cornerstone of chemotherapy for HCC is doxorubicin (Adriamycin, ADA), but doxorubicin resistance prevents these patients from receiving a complete course of treatment. Doxorubicin activates the p53 pathway to cause apoptosis in human HCC cells[222]. In contrast to wild-type p53, which enhances doxorubicin chemosensitivity in the drug-resistant human HCC cell line Bel7402/5-fluorouracil (5-FU), mutant p53 enhances doxorubicin resistance in HCC cell lines[205]. Doxorubicin sensitivity is reduced by p53 activation inhibition[223]. N-myc downstream regulated gene-1, a downstream target of p53, also contributes to doxorubicin resistance in HCC cells[224]. 5-FU and vincristine resistance in HCC cells is linked to p53 mutation and inactivation addition to doxorubicin resistance[218].

For HCC patients, doxorubicin and sorafenib combined therapy produce better results than doxorubicin alone does in terms of median time to progression, overall survival and progression-free survival[103]. These findings suggest that the use of sorafenib in combination with doxorubicin is effective in the treatment of HCC[218]. By reducing Bcl-2 expression, microRNA-34a, a specific target of p53, improves the susceptibility of human HCC cells to the anticancer impact of sorafenib, amplifying sorafenib-induced apoptosis and damage[225]. CDDP, doxorubicin, 5-FU and other treatments are ineffective against HCC cells due to p53 mutation and inactivation, among other factors. The G245D mutation in p53 reduces the chemosensitivity of HCC cells by making them resistant to histone deacetylase inhibitors[218]. Contrarily, dominant-negative p73 (Delta Np73) can suppress p53 function, leading to treatment resistance. Chemoresistance is caused by deltaNp63alpha's direct interference with the p53 family target genes' ability to activate transcription[226]. The mechanism underpinning p53-related chemoresistance may be started or controlled by both extrinsic and intrinsic factors because chemotherapy-induced apoptosis involving p53 in HCC is mediated by extrinsic and intrinsic pathways [218].

p-53 repression leads to doxorubicin resistance in HCC

Dox is the cornerstone of chemotherapy for HCC, but Dox resistance prevents patients with HCC from receiving an effective course of treatment[227]. Dox activates the p53 pathway in human HCC cells to cause apoptosis. Ma *et al*[227] looked at the connection between Lgr5 and p53 expression in HCC in order to clarify the molecular mechanism of Dox resistance[227]. They discovered that Lgr5 stimulates p53 degradation in HCC cell lines and that there is an adverse connection between Lgr5 and p53 expression in HCC tissues. Target gene PDCD5 was found; PDCD5 is a protein involved in apoptosis and is thought to act as a tumour suppressor in a variety of tumour types, particularly lung cancer, breast cancer, and glioma[228,229]. In the presence of DNA damage, PDCD5 can interact with and stabilize p53 to encourage apoptosis[230]. They observed that PDCD5 serves as an independent predictive factor and serves as an antioncogene. Drug resistance caused by Lgr5 is only partially reversed by PDCD5 overexpression. They initially created transfected HCC cell lines that overexpressed Lgr5 and PDCD5, and we discovered that neither protein could enhance the expression of the other at the transcriptional and posttranscriptional stages[227]. This information allowed us to expand our understanding of the interaction between Lgr5 and PDCD5. In addition, they discovered that Lgr5 may

Table 1 List of clinical trials undergoing for hepatocellular carcinoma targeting p53

NCT No.	Title	Drug	Present status	Population	Phase	Date	Location
NCT02418988	Trans-catheter Chemo-embolization combined with rAd-p53 gene injection in treatment of advanced HCC	TACE plus rAd-p53 artery injection	Unknown status	Estimated enrolment: 120 participants. Age: 18 years to 80 years (Adult, older adult)	Advanced adult HCC: Phase 2	Study start study: July 2014. Primary completion study: May 2017. Study completion date: December 2017	China, Shanxi
NCT02509169	Trans-catheter Chemo-embolization combined with p53 gene therapy for treatment of advanced HCC	TAE plus p53 gene	Unknown status	Estimated enrolment: 60 participants. Age: 18 years to 85 years	Advanced HCC: Phase 2	Study start study: October 2014. Primary completion study: December 2016. Study completion date: December 2016	China, Liaoning
NCT02561546	p53 gene therapy in Treatment of diabetes concurrent with HCC	p53 gene therapy	Unknown status	Estimated enrolment: 40 participants. Age: 18 years to 85 years	HCC: Phase 2	Study start study: December 2015. Primary completion study: October 2017. Study completion date: December 2017	China, Liaoning
NCT00003147	Gene therapy in treating patients with cancer of the liver	Ad5CMV-p53 gene	Terminated	Actual enrolment: 30 participants. Age: 18 years and older (Adult, older adult)	Liver cancer: Phase 2	First posted: August 24, 2004. Last Update posted: February 5, 2013	United States, New York. United States, Pennsylvania
NCT02432963	Vaccine therapy and pembrolizumab in treating patients with solid tumours that have failed prior therapy	Modified Vaccinia Virus Ankara Vaccine Expressing p53	Active and recruiting	Actual enrolment: 11 participants. Age: 18 years and older (Adult, older adult)	HCC: Phase 1	First posted: May 4, 2015. Last Update posted: March 21, 2023	United States, California
NCT03544723	Safety and efficacy of p53 gene therapy combined with Immune checkpoint inhibitors in solid tumours	Ad-p53	Unknown status	Estimated enrolment: 40 participants. Age: 18 years and older (Adult, older adult)	Solid tumour: Phase 2	Study start study: October 1, 2018. Primary completion study: June 30, 2022. Study completion date: December 31, 2022	United States, Illinois

bind with PDCD5 in the cytoplasm and stop it from translocating. As a result, PDCD5 was unable to bind to p53 and translocate to the nucleus, stabilizing p53. This suggests that Lgr5 Lowers the effective level of PDCD5 as opposed to the overall concentration of this protein. In HCC cells treated with Dox or even untreated cells, high levels of Lgr5 could lower p53 protein levels and prevent apoptosis[227]. They overexpressed PDCD5 in HCC cells that had been stably transfected with Lgr5 to examine how Lgr5 affected PDCD5. They discovered that this decreased the cells' ability to resist Dox and caused them to undergo apoptosis, which suggests that PDCD5 overexpression is antagonistic to Lgr5. These observations indicated that Lgr5 could prevent PDCD5 from translocating into the nucleus by binding directly to PDCD5 with its N-terminal extracellular domain and causing p53 to degrade. Recent studies demonstrating that PDCD5 may move from the cytoplasm to the nucleus after Dox treatment[231] reinforce this conclusion. Additionally, we discovered that Lgr5 prevents Dox-induced apoptosis in HCC cells *via* inhibiting the p53 pathway[227].

They offer a hypothesis for a brand-new mechanism of Dox resistance in HCC cells, one that emphasizes the crucial function of Lgr5, which is effectively triggered by this chemotherapy medication. When the N-terminal extracellular domain of the triggered Lgr5 directly binds to PDCD5, it inhibits PDCD5's nuclear translocation[227]. This is how the induced Lgr5 functions as a direct negative regulator of PDCD5. The lack of p53 stabilization caused by the decreasing PDCD5 levels within the nucleus causes an EMT-like phenotype, which inhibits apoptosis and eventually results in Dox resistance[227].

CONCLUSION

The recent literature was examined in this review to show the negative impacts of MDM2-p53 axis malfunction on the transformation of hepatoma cells. Despite numerous studies proposing tumour cell-specific signaling pathways that affect the MDM2-p53 axis, the mechanisms causing malfunction in this axis during the progression of HCC are intricate. To better diagnose and treat HCC at an early stage,

future studies should concentrate on the characteristic features of MDM2-p53 axis malfunction in chronic progressive liver diseases that are closely linked to the onset and advancement of HCC. The balance of the MDM2-p53 axis in chronic liver illnesses could be modulated by a number of molecules, and it has been discovered that HCC tissues have severely altered levels of these molecules. By emphasizing on the malfunction of the MDM2-p53 axis during the conversion of healthy hepatocytes into hepatoma cells, we intended to offer some guidance for techniques to cure pre-HCC.

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FOOTNOTES

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Basic Study

- 71 Novel, non-colonizing, single-strain live biotherapeutic product ADS024 protects against *Clostridioides difficile* infection challenge *in vivo*

Murphy CK, O'Donnell MM, Hegarty JW, Schulz S, Hill C, Ross RP, Rea MC, Farquhar R, Chesnel L

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Basic Study

Novel, non-colonizing, single-strain live biotherapeutic product ADS024 protects against *Clostridioides difficile* infection challenge *in vivo*

Christopher K Murphy, Michelle M O'Donnell, James W Hegarty, Sarah Schulz, Colin Hill, R Paul Ross, Mary C Rea, Ronald Farquhar, Laurent Chesnel

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Abstract

BACKGROUND

The Centers for Disease Control and Prevention estimate that *Clostridioides difficile* (*C. difficile*) causes half a million infections (CDI) annually and is a major cause of total infectious disease death in the United States, causing inflammation of the colon and potentially deadly diarrhea. We recently reported the isolation of ADS024, a *Bacillus velezensis* (*B. velezensis*) strain, which demonstrated direct *in vitro* bactericidal activity against *C. difficile*, with minimal collateral impact on other members of the gut microbiota. In this study, we hypothesized that *in vitro* activities of ADS024 will translate *in vivo* to protect against CDI challenge in mouse models.

AIM

To investigate the *in vivo* efficacy of *B. velezensis* ADS024 in protecting against CDI challenge in mouse models.

METHODS

To mimic disruption of the gut microbiota, the mice were exposed to vancomycin prior to dosing with ADS024. For the mouse single-dose study, the recovery of ADS024 was assessed *via* microbiological analysis of intestinal and fecal samples

at 4 h, 8 h, and 24 h after a single oral dose of 5×10^8 colony-forming units (CFU)/mouse of freshly grown ADS024. The single-dose study in miniature swine included groups that had been pre-dosed with vancomycin and that had been exposed to a dose range of ADS024, and a group that was not pre-dosed with vancomycin and received a single dose of ADS024. The ADS024 colonies [assessed by quantitative polymerase chain reaction (qPCR) using ADS024-specific primers] were counted on agar plates. For the 28-d miniature swine study, qPCR was used to measure ADS024 levels from fecal samples after oral administration of ADS024 capsules containing 5×10^9 CFU for 28 consecutive days, followed by MiSeq compositional sequencing and bioinformatic analyses to measure the impact of ADS024 on microbiota. Two studies were performed to determine the efficacy of ADS024 in a mouse model of CDI: Study 1 to determine the effects of fresh ADS024 culture and ADS024 spore preparations on the clinical manifestations of CDI in mice, and Study 2 to compare the efficacy of single daily doses *vs* dosing 3 times per day with fresh ADS024. *C. difficile* challenge was performed 24 h after the start of ADS024 exposure. To model the human distal colon, an anaerobic fecal fermentation system was used. MiSeq compositional sequencing and bioinformatic analyses were performed to measure microbiota diversity changes following ADS024 treatment. To assess the potential of ADS024 to be a source of antibiotic resistance, its susceptibility to 18 different antibiotics was tested.

RESULTS

In a mouse model of CDI challenge, single daily doses of ADS024 were as efficacious as multiple daily doses in protecting against subsequent challenge by *C. difficile* pathogen-induced disease. ADS024 showed no evidence of colonization based on the observation that the ADS024 colonies were not recovered 24 h after single doses in mice or 72 h after single doses in miniature swine. In a 28-d repeat-dose study in miniature swine, ADS024 was not detected in fecal samples using plating and qPCR methods. Phylogenetic analysis performed in the human distal colon model showed that ADS024 had a selective impact on the healthy human colonic microbiota, similarly to the *in vivo* studies performed in miniature swine. Safety assessments indicated that ADS024 was susceptible to all the antibiotics tested, while *in silico* testing revealed a low potential for off-target activity or virulence and antibiotic-resistance mechanisms.

CONCLUSION

Our findings, demonstrating *in vivo* efficacy of ADS024 in protecting against CDI challenge in mouse models, support the use of ADS024 in preventing recurrent CDI following standard antibiotic treatment.

Key Words: Gut; Single-strain live biotherapeutic product; ADS024; *Clostridioides difficile*; *Clostridioides difficile* challenge model; Microbiota

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Core Tip: *Clostridioides difficile* (*C. difficile*), a Gram positive pathogen associated with life-threatening gastrointestinal disease, colonizes millions of healthy people worldwide, causing disease in individuals with disrupted gut microbiomes. Here, we demonstrate *in vivo* efficacy of recently isolated *Bacillus velezensis* ADS024, without colonization, in protecting against *C. difficile* infection (CDI) challenge in mouse models. We also show that this novel strain has minimal effects on the gut microbiome in a human distal colon model and miniature swine. This study supports further investigation of ADS024 as a single-strain, live biotherapeutic product candidate for preventing recurrent CDI following successful standard-of-care antibiotic therapy.

Citation: Murphy CK, O'Donnell MM, Hegarty JW, Schulz S, Hill C, Ross RP, Rea MC, Farquhar R, Chesnel L. Novel, non-colonizing, single-strain live biotherapeutic product ADS024 protects against *Clostridioides difficile* infection challenge *in vivo*. *World J Gastrointest Pathophysiol* 2023; 14(4): 71-85

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INTRODUCTION

Clostridioides difficile (*C. difficile*; previously *Clostridium difficile*) is an opportunistic Gram positive pathogen that takes advantage of a disrupted gut microbiome to cause potentially life-threatening gastrointestinal (GI) disease[1,2]. *C. difficile* infects millions of people worldwide, resulting in a range of pathologies, from mild abdominal symptoms to potentially life-threatening colitis. It is the most frequently identified health care-related infection in the United States, where community-acquired infections are increasing[3]. Risk factors include prior hospitalization, residence in a nursing home care facility, advanced age, underlying disease, and alterations in the gut microbiota[3-5]. *C. difficile* infection (CDI) is mainly characterized by diarrhea, but the spectrum of disease can progress to colitis and toxic megacolon[6]. The

pathogenicity of *C. difficile* is mainly mediated by 2 exotoxins, toxin A (TcdA) and toxin B (TcdB)[2]. In addition, certain strains of *C. difficile* may also produce a binary toxin called *C. difficile* transferase[2]. The clinical symptoms described above are mediated by TcdA and TcdB, which precipitate an inflammatory response and cytotoxicity in epithelial cells of the GI tract[2]. While the relative contributions of TcdA and TcdB may be specific to the host species[7,8], TcdB is the predominant mediator of CDI[7,9] and the most clinically relevant[8].

Recurrent CDI (rCDI) following initial successful antibiotic treatment remains a clinical challenge, with each episode associated with an increased risk for recurrence (25% after the first, 45% after the second, and 65% after the third and subsequent episodes), creating a cycle of infection and antibiotic use[10,11]. The causes of rCDI are primarily attributed to the failure of antibiotic-disrupted gut microbiota to restrict the germination and subsequent overgrowth of surviving spores of *C. difficile* and an altered immune response as a result of the action of the *C. difficile* toxins[10,12]. Therefore, predicting future recurrence is crucial. Machine learning is emerging as a useful method of predicting outcomes by comparing changes in fecal microbial composition and metabolome in patients with rCDI *vs* those without[12,13].

The gut microbiome plays a critical role in preventing CDI among its many functions. Indeed, perturbation of the microbiome through frequent antibiotic administration is a critical risk factor for subsequent recurrences, presumably as a result of the time needed to recover a diverse and protective microbiota. Even with short-term antibiotic treatment, microbiota recovery takes several months[14], and perturbations can persist up to 4 years post treatment[15,16]. In addition, the standard treatment of initial and rCDI often involves broad-spectrum antibiotics, including vancomycin[1]. Even though CDI clinical cure rates are high[17,18], patients remain at risk for recurrence[18]. Thus, degrading TcdA and TcdB, killing germinating *C. difficile* spores, and reducing inflammation are vital for restoring protective intestinal microbiota and successfully preventing recurrent episodes of CDI.

In a recent study, we described the isolation of ADS024, a *Bacillus velezensis* (*B. velezensis*) strain in development as a single-strain live biotherapeutic product (SS-LBP) to prevent the recurrence of CDI after clinical cure by standard antibiotic treatment[19]. LBPs are biological medicinal products containing live microorganisms as active substances. They are being studied to prevent, treat, or cure various diseases[20–24], including allergies, dental disorders, and GI, dermatologic, and gut-brain-axis-related conditions[25]. We demonstrated that ADS024 has bactericidal activity against *C. difficile*, with minimal impact on other common members of the gut microbiota, and produces proteases that can degrade both TcdA and TcdB[19]. The current study expands on our previous findings by demonstrating the protective effects of ADS024 in a mouse model of CDI, the minimal impact of ADS024 on the gut microbiome of miniature swine and humans, and its lack of colonization/engraftment in miniature swine and mouse models. The results of these studies support further development of ADS024 as an SS-LBP for the prevention of rCDI after clinical cure following completion of standard antibiotic treatment.

MATERIALS AND METHODS

Ethics statement

Biological samples for this work were collected using Clinical Research Ethics Committee, Cork, Ireland, approved consents for the Protocol “ControlMET” numbered APC065.

Animal care and use statement

The protocol and any amendment(s) or procedures involving the care and use of animals in this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at NeoSome Life Sciences. During the study, the care and use of animals were conducted with guidance from the United States National Research Council guidelines. Veterinary care was available throughout the study for animals to be examined by the veterinary staff as warranted by clinical signs or other changes. There were no changes in the animals that required veterinary intervention to ameliorate pain or distress.

Quantification of ADS024 in mice (single-dose study)

The recovery of ADS024 was assessed in intestinal and fecal samples of mice at various times after a single dose of freshly grown ADS024. Prior to dosing with ADS024, the mice were exposed to vancomycin to disrupt their microbiota in a clinically relevant manner. Further detail is provided in the [Supplementary material](#).

Quantification of ADS024 in minipigs (single-dose study)

The aim of this study was to investigate ADS024 fecal content from male miniature swine following a single dose of ADS024 capsules over the time course of 5 d post ADS024 dosing. Further detail is provided in the [Supplementary material](#).

In vivo mouse model of *C. difficile* infection

The study design was adapted from Chen *et al*[26] and modified to test if prior dosing with ADS024 could protect against subsequent challenge by *C. difficile*. Accordingly, the ADS024 treatment started 24 h prior to the CDI (spore) challenge. Upon their receipt at NeoSome Life Sciences, the mice, while not tested for *C. difficile* colonization/infection prior to inoculation with *C. difficile*, were examined by personnel to ensure acceptable health status. Veterinary care was provided by the veterinarians and staff employed by NeoSome Life Sciences. The mice were acclimated for at least 5 d prior to use. Following acclimation, the mice were infected with *C. difficile* strain VPI10463 (6.30×10^5 to 1.97×10^6 CFU, a higher dose

compared with the 10^2 to 10^5 CFU in the paper by Chen *et al* [26]), and 65 and 40 mice were treated with ADS024 or saline, respectively, across the conducted studies. The mice were then monitored for survival, weight loss, and disease-related clinical observations, including wet tail, diarrhea, hunched posture, dehydration, and lethargy. The daily score of adverse health observations was calculated based on criteria specified in [Supplementary Table 1](#). Further detail, including antibiotic pretreatment, is provided in the [Supplementary material](#).

Distal colon model

The micro-Matrix (Applikon Biotechnology, Delft, Netherlands) was used to model the human distal colon and examine the impact of ADS024 on gut microbial populations. The anaerobic fecal fermentation model of the distal colon has been previously described [27]. Further detail is provided in the [Supplementary material](#).

MiSeq compositional sequencing and bioinformatic analysis: All extracted samples from the distal colon model were prepared for MiSeq DNA compositional sequencing, and 16S data were then organized by alpha diversity, beta diversity, and phylum and genus diversity. Further detail is provided in the [Supplementary material](#).

Statistical significance determination: ADS024-treated wells were then assessed by comparing differences to wells with untreated media. Further detail is provided in the [Supplementary material](#).

Quantitative polymerase chain reaction: Absolute quantification by quantitative polymerase chain reaction (qPCR) was performed to determine total bacterial numbers and specific populations from fecal samples. Further detail is provided in the [Supplementary material](#).

ADS024 quantification in miniature swine fecal samples, 28-d study

To determine the impact of ADS024 administration on the gut composition of miniature swine, qPCR, MiSeq compositional sequencing, and bioinformatic analysis were performed in duplicate using fecal samples. Further detail is provided in the [Supplementary material](#).

ADS024 susceptibility to antibiotics

Two batches each of lyophilized drug substances (CO-33-10A2 and CO-33-12A2) and frozen glycerol stocks [research cell bank (CO-33 ART24BHI) and the master cell bank (CO-33 ART24SYD)] were tested for antimicrobial susceptibility using the reference broth microdilution method per the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI M07 2018). Tests with metronidazole were also carried out under anaerobic conditions (CLSI M11 2018). Minimum inhibitory concentration (MIC) values obtained against ADS024 were validated by concurrently testing CLSI-recommended ATCC quality control reference strains (CLSI M100 2019). Categorical interpretations for the MIC results obtained against ADS024 used CLSI M45 (2015) [28] breakpoint criteria or European Food Safety Authority (EFSA 2012) [29] microbiological cutoff values.

Potential virulence factors and antimicrobial resistance genes of ADS024 were analyzed using IslandViewer 4. The annotated ADS024 genome was first analyzed using IslandViewer 4, and a separate web server was subsequently used to interpret the data. The output was based on mapping as detailed in [Supplementary Figure 1](#) and stored as a Microsoft Excel spreadsheet that contained the output of the integrated IslandPath-DIMOB, SIGI-HMM, and IslandPick prediction methods.

Biostatistics statement

The bioinformatics and statistical analyses relating to 16S sequencing and the micro-Matrix, as described in this manuscript, were verified by Dr. Michelle M. O'Donnell of APC Microbiome Ireland, University College Cork. The bioinformatics and statistical analyses relating to all animal-related research described in this paper were verified by Dr. Christopher Murphy of Adiso Therapeutics, Inc.

RESULTS

ADS024 is recovered in fecal samples after single-dose administration in mice and miniature swine

Microbiological analysis was conducted on fecal pellets and GI tissue from mice pre-dosed with vancomycin to mimic disruption of the gut microbiota. The mice were then administered a single oral dose of approximately 5×10^8 colony-forming units (CFU)/mouse of ADS024. ADS024 has a very distinctive morphology allowing for visual detection on agar plates [19]. ADS024 colonies were recovered in feces following plating on agar media at 4 h, 8 h, and 24 h post dose ([Figure 1A](#)). No ADS024 colonies were detected in feces after the 24-h post-dose time point. Only 1 GI tissue sample contained detectable amounts of ADS024, present in the upper intestine 24 h after ADS024 administration ([Supplementary Figure 2](#)).

The results from a single-dose study in minipigs (3 male miniature swine per group) were consistent with those from the mouse studies, showing a lack of detection in fecal and tissue samples past 72 h, supporting daily dosing for clinical use. The single-dose study in miniature swine included groups that had been pre-dosed with vancomycin to induce disruption of the gut microbiota, as described under Materials and Methods, and that had been exposed to a dose range of ADS024 (Groups 2, 3, and 4 received 6.2×10^7 , 7.1×10^8 , or 7.1×10^9 CFU, respectively), and a group that was not pre-dosed with vancomycin and received a single dose of ADS024 (Group 5 received 7.1×10^9 CFU). The control group, group

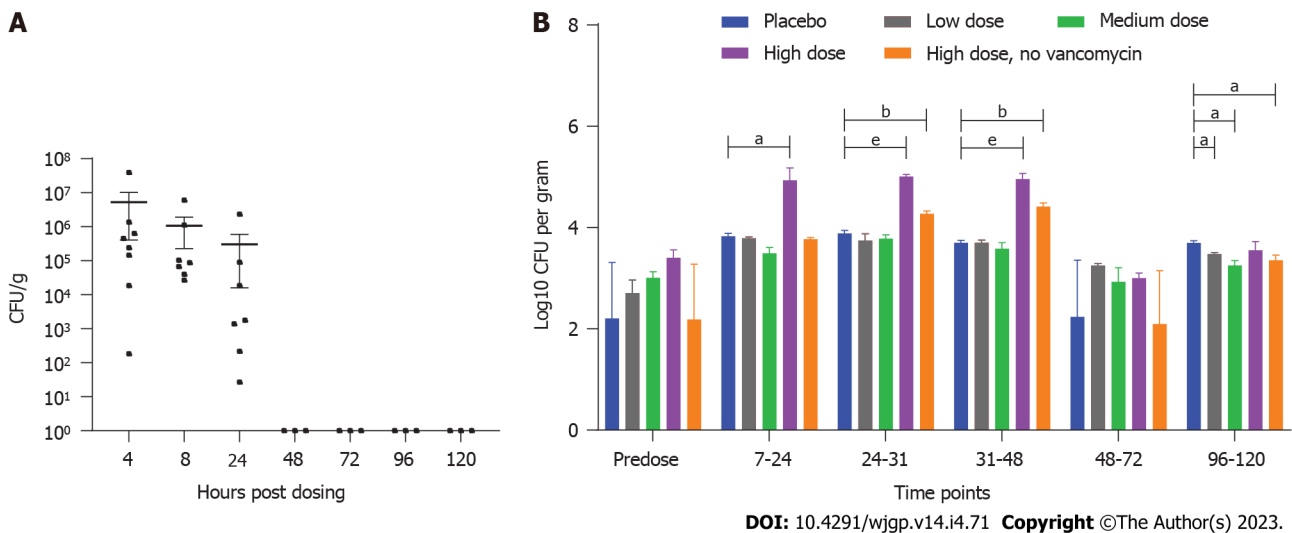


Figure 1 Quantification of ADS024 (fresh culture) in the mice and miniature swine fecal samples for the single-dose study. A: The mice were predosed with vancomycin to mimic disruption of gut microbiota and administered a single oral dose of 5×10^8 colony-forming units (CFU)/mouse of ADS024. Microbiological analysis was subsequently conducted on the mouse fecal pellets and gastrointestinal tissue. The ADS024 colonies were recovered in feces following plating on agar media at 4 h, 8 h, and 24-h post dose; B: The single-dose study in miniature swine included 3 groups predosed with vancomycin to induce disruption of gut microbiota. They were exposed to the following dose range of ADS024: Group 2 (low dose) 6.2×10^7 CFU; Group 3 (medium dose) 7.1×10^8 CFU; and Group 4 (high dose) 7.1×10^9 CFU. There was 1 group not pre-dosed with vancomycin but exposed to a single dose of ADS024: Group 5 (no vancomycin, high dose) 7.1×10^9 CFU. The control group (Group 1, placebo) did not receive vancomycin or ADS024. After single oral administration of ADS024 to the miniature swine, the ADS024 colonies, assessed by quantitative polymerase chain reaction (qPCR), were detected on agar plates in the ADS024-treated Groups 4 and 5, regardless of vancomycin therapy, at colony counts higher than placebo controls that displayed only *Bacillus*-like colonies and not ADS024-specific ones based on qPCR, at the 7-h to 48-h time points. *P* value significance: ^a $P \leq 0.05$, ^b $P \leq 0.01$, ^c $P \leq 0.001$.

1, did not receive vancomycin or ADS024. After single oral administration of ADS024 in miniature swine, the ADS024 colonies (assessed by qPCR using ADS024-specific primers) were detected on agar plates in the ADS024-treated Groups 4 and 5, regardless of vancomycin therapy, at colony counts higher than placebo controls (which grew only *Bacillus*-like, but not ADS024-specific, colonies) at the 7- to 48-h time points (Figure 1B). The qPCR data (Supplementary Figure 3) confirmed the plating colony counts (Figure 1B), as no ADS024-selective agar plates were available.

ADS024 colonies were considered to have increased if the values were higher than the highest predose value of 10^4 CFU/g and higher than the corresponding placebo value for the given time point. The 7- to 48-h post-dose period was the Discovery Window, and this term was used for subsequent qPCR analysis. The qPCR analysis of Groups 2 to 5 showed that ADS024 was present in the Discovery Window samples at 24 h to 31 h post dose for the low-dose Group 2, 24 h to 48 h for the mid-dose Group 3, and 7 to 48 h for the high-dose Groups 4 and 5 at variable concentrations higher than predose in all animals (except for the Group 5 samples) (Figure 1B).

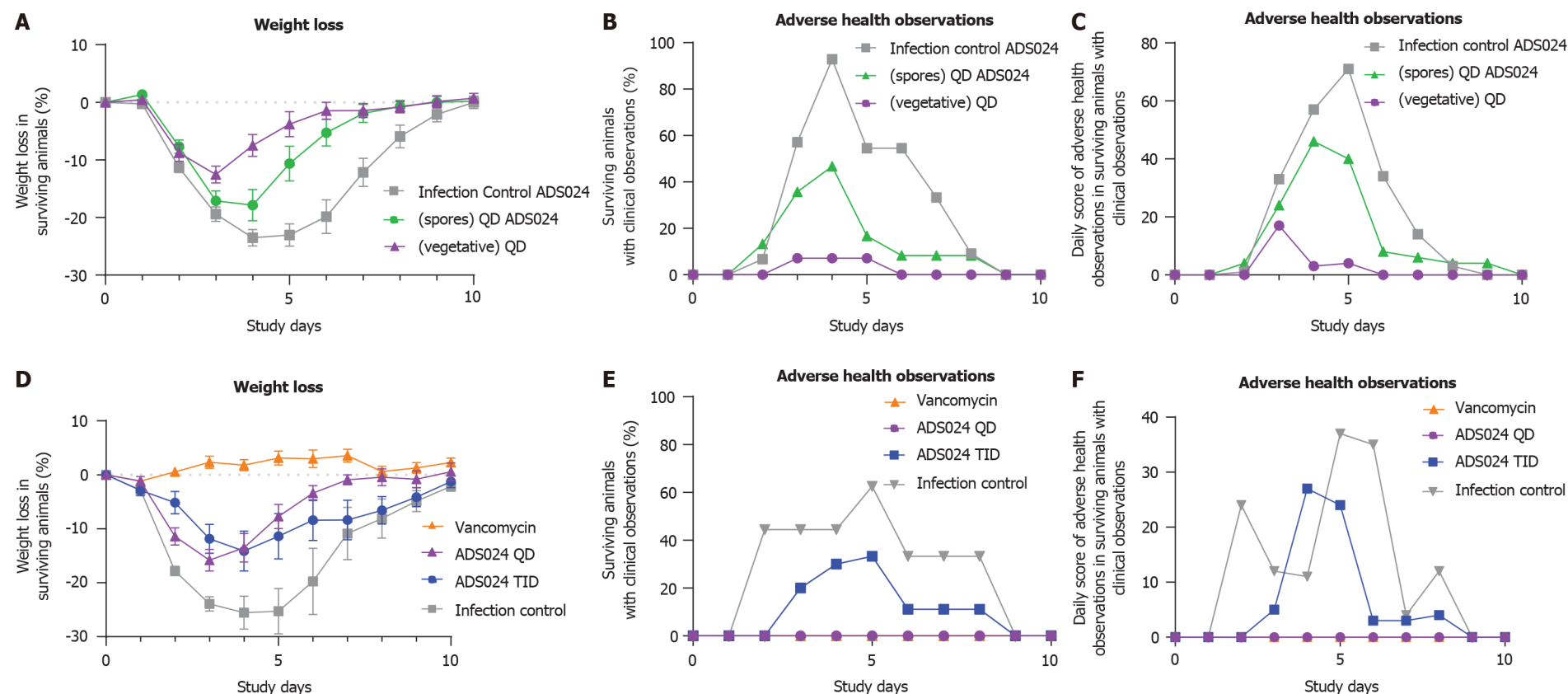
ADS024 is efficacious in a mouse model of CDI

To confirm if the toxin degradation and *C. difficile*-killing activities observed in our *in vitro* studies[19,20] translated to *in vivo* efficacy, we utilized a mouse model of CDI. The animal model was adapted from Chen *et al*[26] as described under Materials and Methods, and the *C. difficile* challenge was performed 24 h after the start of ADS024 exposure.

Two studies were performed to determine the efficacy of ADS024 in the mouse model of CDI. The first study (Study 1, Figure 2A-C) determined the effects of fresh ADS024 culture and ADS024 spore preparations on the clinical manifestations of CDI in mice, namely weight loss (Figure 2A) and adverse clinical features (Figure 2B and C). The second study (Study 2, Figure 2D-F) compared the efficacy of single daily doses (QD) *vs* dosing 3 times per day (TID) with fresh ADS024.

Study 1: ADS024 was delivered to mice ($n = 15$ per group) as resuspended spore suspensions [in phosphate-buffered saline (PBS)] or as a freshly prepared daily culture (ADS024 bacteria resuspended in PBS). The placebo (infection control) group demonstrated an average maximum weight loss of 24.4%, and the mice experienced adverse health effects for 33% of the days (Figure 2A-C). In comparison, mice that received ADS024 as a fresh culture suffered less weight loss compared with the infection (vehicle-dosed) controls (13.5% maximum average percentage of pre-infection weight loss; Figure 2A), and the mice experienced adverse health effects for only 3% of the days (Figure 2B), which was the lowest daily score of adverse health observations among the 3 groups (Figure 2C). The ADS024 spore preparation-treated mice exhibited a maximum weight loss of 20% on day 4, and for 19% of the days, the mice scored adverse health effects (Figure 2A-C). Therefore, mice treated with fresh ADS024 had lower morbidity and mortality than those treated with ADS024 spores or mice that received only placebo.

Study 2: Dose frequencies of QD and TID were compared in mice ($n = 10$ per group). The untreated (infection control) group had an average maximum weight loss of 25% on day 4 (Figure 2D), similar to observations in Study 1. In



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Figure 2 *Clostridioides difficile* infection challenge mouse model studies. Study 1: The effects of fresh (vegetative) ADS024 culture vs ADS024 spore preparations on the clinical manifestations of *Clostridioides difficile* infection (CDI) over 10 d. ADS024 was delivered to the mice ($n = 15$ per group) as resuspended spore suspensions [in phosphate-buffered saline (PBS)] or as a freshly prepared daily culture (ADS024 bacteria resuspended in PBS) 24 h prior to *C. difficile*. A: The weight loss comparison between mice receiving vegetative vs spore ADS024 relative to the infection (vehicle-dosed) controls; B: The adverse health effects comparison in surviving animals among the 3 groups (control, vegetative ADS024, and spore ADS024); C: The daily score of adverse health observations among the 3 groups was determined as shown in [Supplementary Table 1](#). Study 2: The effects of single daily (QD) doses vs 3 times daily (TID) administration with fresh ADS024 in the mice ($n = 10$ per group) over the course of 10 d; D: The weight loss comparison among the 4 groups (vancomycin, untreated, QD, and TID); E: The adverse health effects comparison in the surviving animals among the 4 groups; F: The daily score of adverse health observations among the 4 groups was determined as shown in [Supplementary Table 1](#).

comparison, mice that received ADS024 QD had an average maximum weight loss of 16% on day 3 (from the pre-dose weight) before recovering to the group average pre-infection body weight at day 10 (Figure 2D). Mice receiving ADS024 TID demonstrated a maximum weight loss of 14% on day 4 (Figure 2D), similar to that in Study 1. The vancomycin control mice had no weight loss throughout the study (Figure 2D). The ADS024 QD and vancomycin groups did not display adverse clinical features, as demonstrated by overlapping values in Figure 2E and F. In contrast, the ADS024 TID

group did display adverse clinical features (Figure 2E and F), perhaps due to the TID oral gavage-related stress. Therefore, there was no obvious benefit from repeat dosing because the efficacy in this model was similar; furthermore, there were fewer adverse health events in the QD-dosed group than in the TID-dosed group.

ADS024 selectively impacts the gut microbiome in an *in vitro* model of the human distal colon

We used anaerobic fecal fermentation to model the distal colon as previously described[27]. qPCR was used to analyze the effect of ADS024 exposure on 5 different components of human colonic microbiota (Enterobacteriaceae, Bacillota, Bacteroidota, Verrucomicrobiota, and *Lactobacillus*) and to determine the number of ADS024 colonies in inoculated wells (Figure 3A and B). ADS024 was detectable in the ADS024-inoculated wells (Figure 3A). Still, its concentration did not increase during the experiment and had a negligible impact on other gut microbiota components after 24 h of incubation (Figure 3B).

MiSeq 16S (Illumina, San Diego, CA, United States) compositional sequencing analyses at the phylum level (Figure 3C and Supplementary Table 2) revealed an increase in Actinomycetota (approximately 13%; $P < 0.0001$) and significant decreases in Bacillota (approximately 12%; $P < 0.01$), Fusobacteriota (approximately 0.001%; $P < 0.05$), and Verrucomicrobiota (approximately 0.4%; $P < 0.05$) compared with media-only treatment. Compositional analyses at the genus level (Figure 3D and Supplementary Table 3) revealed 12 genera showing significant differences after 24 h of treatment compared with the media-only controls. Among the 7 genera positively impacted by the exposure to ADS024, increases in *Bifidobacterium* (approximately 12%; $P < 0.00003$) and *Bacteroides* (approximately 2%; $P < 0.05$) were found compared with media-only treatment (Figure 3D). These results correlate with the MiSeq compositional sequencing and bioinformatic analyses that were performed to measure microbiota diversity changes following ADS024 treatment using Shannon and Simpson indexes for alpha diversity (Figure 4A and B) and UniFrac principal coordinates analysis (PCoA) for beta diversity (Figure 4C). After 24 h of incubation, ADS024 had less effect on alpha diversity than the media-only controls relative to baseline (Figure 4A and B). The microbial diversity between the media-exposed and ADS024-exposed samples was similar in beta diversity changes (Figure 4C).

Miniature swine gut microbiota is not impacted after 28-d dosing with ADS024

ADS024 Levels from fecal samples were determined by qPCR after QD oral administration of ADS024 capsules containing 5×10^9 CFU in male and female miniature swine for 28 consecutive days as described in Materials and Methods (Supplementary Figure 4). ADS024-specific genetic material was below the limit of detection in both the placebo and ADS024-dosed groups at all assayed time points. Subsequently, MiSeq compositional sequencing and bioinformatic analyses were performed to measure the impact of ADS024 on microbiota in miniature swine fecal samples collected pre dose (day 15), on the last day of dosing (day 29), and 14 d after the last dose (recovery) using Shannon and Simpson indexes for alpha diversity (Figure 5A and B) and UniFrac PCoA for beta diversity (Figure 5C). The alpha diversity analyses indicated no significant differences in microbiota diversity between the ADS024 and placebo treatment groups at any time (Figure 5A and B). Beta diversity using PCoA plots, based on weighted UniFrac distance matrixes, indicated minor, non-significant differences between the placebo and ADS024 groups (permutational multivariate analysis of variance statistical test in R: $P = 0.617$), and all samples tended to cluster together regardless of treatment or time point (Figure 5C). At the phylum level, no significant differences were detected in the most abundant phyla (Bacillota, Bacteroidota, and Spirochaetota) between treatment groups across time points (Figure 6A). At the genus level, no significant differences were observed across the most abundant genera (*Lactobacillus* and *Streptococcus*) at any time point (Figure 6B).

ADS024 is susceptible to a variety of antibiotics and lacks antimicrobial resistance genes

To assess the potential of ADS024 to be a source of antibiotic resistance, its susceptibility to 18 different antibiotics was tested. The frozen glycerol stock and lyophilized forms of ADS024 samples were assessed as described under materials and methods.

Table 1 summarizes the MIC results for the 18 antimicrobials tested against one lot of ADS024 (4 lots were tested with similar results). All antimicrobial agents showed low MIC values when tested against ADS024, except for metronidazole (MIC ≥ 64 mg/L, depending on atmospheric conditions). ADS024 was susceptible to all 12 drugs tested for which interpretive criteria are available[28,29].

To determine the possible presence of virulence factors or antimicrobial resistance genes, the computational genomic prediction tool IslandViewer 4 was used. Corroborating statistical analyses already published[19], no virulence factors, homologs, or operons for antibiotic resistance were identified, pointing to the low potential of ADS024 to expose the gut microbiota to virulence and antibiotic-resistance genes after oral dosing (Supplementary Figure 1).

DISCUSSION

B. velezensis ADS024, isolated from a human fecal sample, is a member of the operational group *Bacillus amyloliquefaciens*, consisting of the soilborne *B. amyloliquefaciens* and the plant-associated *Bacillus siamensis* and *B. velezensis*. Most often, *B. amyloliquefaciens* is used in literature to describe the operational group *B. amyloliquefaciens* and refers to all three closely related species[30]. In this study, we extended our *in vitro* characterization of ADS024 to show its protective effects against CDI challenge in mouse models. We demonstrated a lack of impact on the gut microbiota composition in human feces and miniature swine models. Our model aligned with a CDI challenge rather than a recurrent model. The animals used in our study were not subjected to an earlier round of CDI or pre-treated with antibiotics, which would damage

Table 1 Antimicrobial susceptibility profiles of ADS024

Antimicrobial	MIC in mg/L (interpretation) for ADS024
	CO-33 ADS024 SYD
Ampicillin	≤ 0.03 (S)
Chloramphenicol	2 (S)
Ciprofloxacin	≤ 0.03 (S)
Clindamycin	0.25 (S)
Erythromycin	0.12 (S)
Fidaxomicin	4
Gentamicin	≤ 0.06 (S)
Kanamycin	≤ 0.50 (S)
Linezolid	0.5
Metronidazole (aerobic)	> 64.00
Metronidazole (anaerobic)	64
Neomycin	≤ 0.12
Penicillin	0.06 (S)
Quinupristin-dalfopristin	2
Rifampicin	0.25 (S)
Streptomycin	2 (S)
Tetracycline	2 (S)
Trimethoprim	≤ 0.25
Vancomycin	≤ 0.25 (S)

Four samples of ADS024 were tested. Shown here are the results using ADS024 from the master cell bank (CO-33 ART24 SYD; frozen). MIC: Minimum inhibitory concentration; S: Susceptible.

their GI tract microbiota, the 2 conditions associated with CDI recurrence.

Previous studies have investigated the *B. amyloliquefaciens* operational group in a mouse model as a prophylactic treatment for *C. difficile*. In one such study by Geeraerts *et al*[21], using C57BL/6J mice and *C. difficile* strain VPI 10463, it was demonstrated that *C. difficile* toxin A and B levels were significantly lower in *B. amyloliquefaciens*-treated mice compared with untreated ones[21]. In addition, a significantly lower extent of colonic tissue damage was observed for *B. amyloliquefaciens*-treated mice compared with the untreated cohort. Consistent with these findings, ADS024 conferred protection against CDI and its clinical manifestations.

Although not directly tested in the experiments shown in this study, one possibility is that the pharmacologic effect of ADS024 was due to its bactericidal activity against *C. difficile* as well as its proteolytic degradation of TcdA and TcdB[19], resulting in fewer observed clinical symptoms. Furthermore, an anti-inflammatory role of *Bacillus*-based probiotics has been demonstrated[20,31]. To this end, the *Bacillus*-based probiotic, Bs 29784, decreased pro-inflammatory signals in a Caco-2 cell model for intestinal epithelia, prevented IkBα degradation, and reduced upregulation of iNOS protein levels, highlighting intestinal anti-inflammatory capabilities[31]. Similarly, ADS024 inhibited toxin B-mediated apoptosis and tissue injury in human colonic epithelial cells and colonic explants[20]. In addition, restoring the host gut microbiota to preexisting conditions plays an equally critical role in recovering from CDI and preventing recurrent infection after the completion of standard antibiotic treatment. We hypothesize that the selective impact of ADS024 on the human microbiota may therefore allow recovery of the normal host microbiota and prevent rCDI in clinical settings.

The aim of the mouse model (Figure 2) was to evaluate whether prior dosing with ADS024 could protect against subsequent challenge by *C. difficile*. We found that single daily (QD) doses of ADS024 were as efficacious as multiple daily (TID) doses in protecting against pathogen-induced disease (Figure 2), and, as reported in the result section of the Study 2 Mouse model of CDI, there was no significant benefit in the TID-dosed group (Figure 2D-F). While there was a slight improvement in the weight loss category in the TID-dosed group compared with the QD-dosed group (14% vs 16%), both groups showed improvement compared with the untreated group (25%) (Figure 2D). In contrast, the adverse clinical features were present only in the TID-dosed group (Figure 2E and F), possibly due to the added stress in the animals from repeated oral gavage[32], which likely negated any positive effect of ADS024. Stress would likely increase assessed disease markers if there were an infected placebo arm with the same dosing regimen.

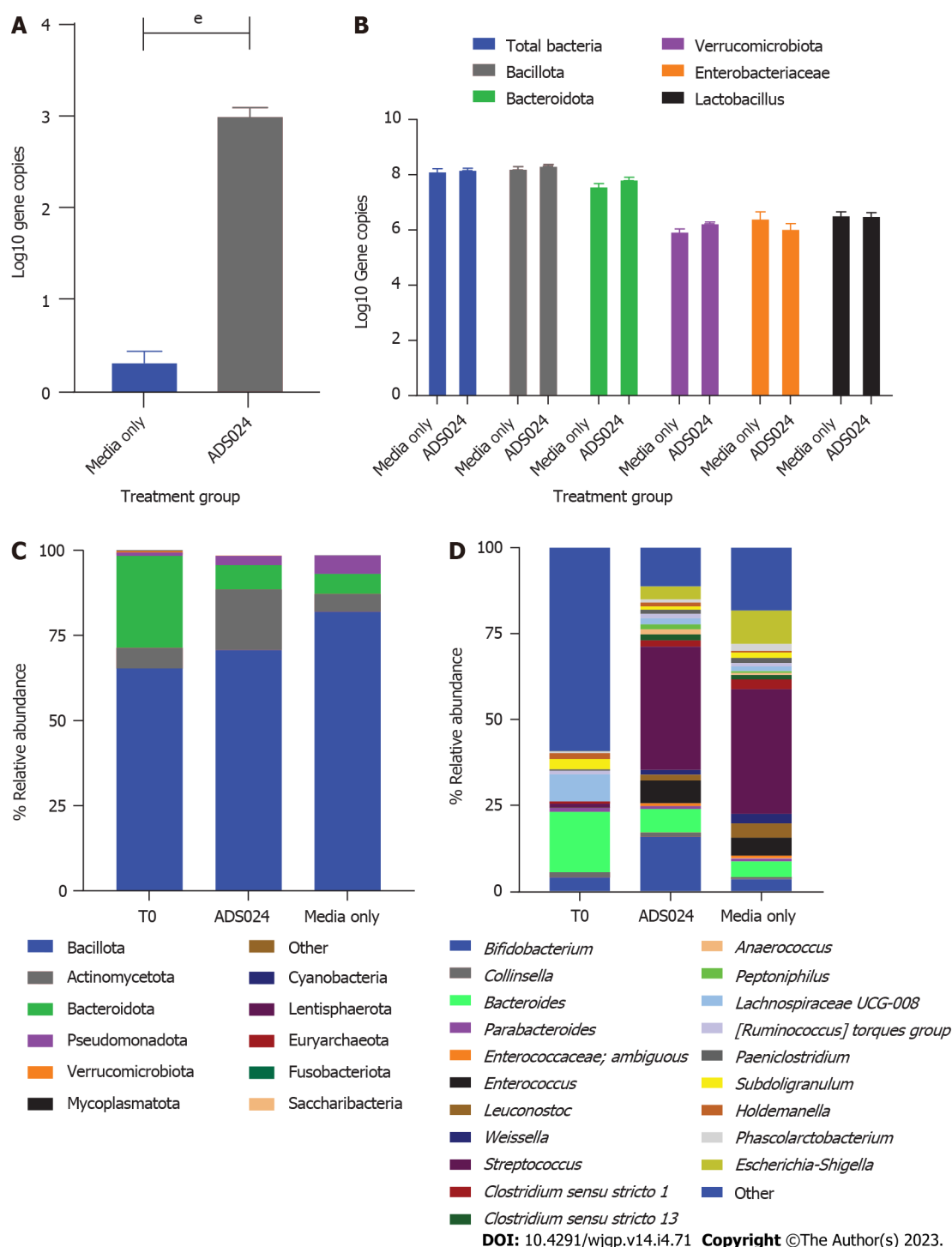


Figure 3 Distal colon model. A: Comparison of the wells inoculated with ADS024 vs media alone was done via quantitative polymerase chain reaction (qPCR) using specific primers to detect ADS024 as described in Materials and Methods; B: qPCR was performed to study the antimicrobial activity of ADS024 on 5 different phyla of the human colonic microbiota; C: MiSeq compositional sequencing analysis was performed to compare the impact of ADS024 on the gut microbiota at the phylum level with that of media alone; D: MiSeq was used to determine the ADS024 effect after 24 h of treatment at the genus level. * $P \leq 0.001$.

ADS024 showed no evidence of colonization (Figure 1) based on the observation that the ADS024 colonies were not recovered 24 h after single doses in mice or 72 h after single doses in minipigs. This suggests that the ADS024-mediated protection against *C. difficile* pathogenesis occurred independently of the colonization of ADS024 in the animals' GI tracts.

ADS024 was also assessed in a 28-d repeat-dose study in miniature swine (Figure 5 and 6), a species that offers a more clinically relevant model of the human GI tract[33]. ADS024 was not detected in fecal samples using plating and qPCR methods (Supplementary Figure 4), which again suggests that ADS024 did not colonize the gut following long-term treatment exposures. To achieve reproducible results with microbial therapeutics in humans, it may be advantageous to use transitory, non-colonizing strains[34], such as ADS024.

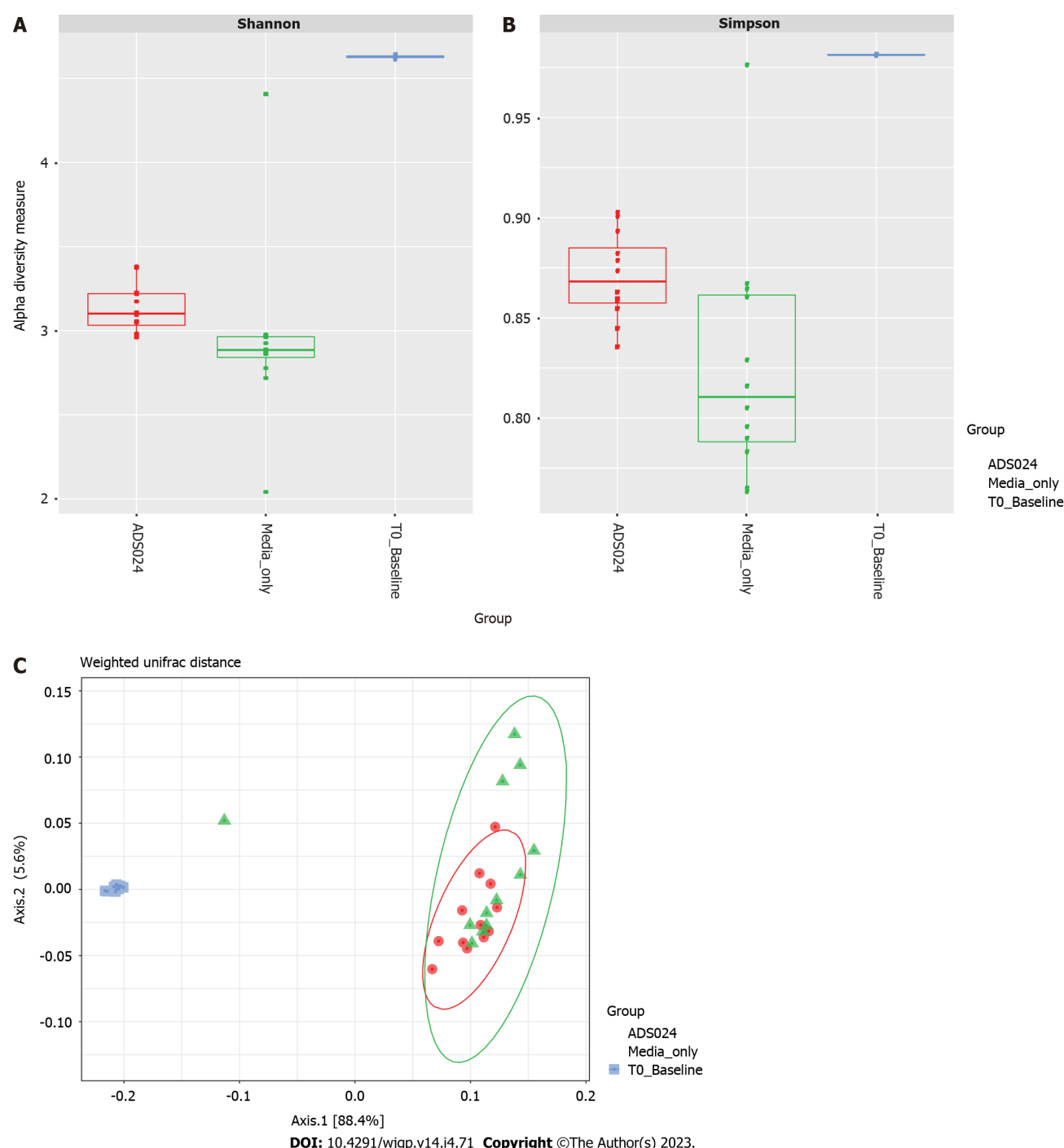


Figure 4 Distal colon model: Alpha and beta diversity analyses. MiSeq compositional sequencing and bioinformatic analysis were performed to measure the microbiota diversity changes following ADS024 treatment using the: A: Shannon index for alpha diversity; B: Simpson diversity index for alpha diversity; C: UniFrac principal coordinates analysis for beta diversity.

Restoring the resident host microbiota is an important factor in preventing rCDI. Thus, the effect of ADS024 on gut microbial composition was investigated using a human distal colon model (Figures 3 and 4). *Ex vivo* systems replicating the distal colon environment have been used to investigate the effect of antimicrobial-producing bacterial strains on the human gut microbiota[35]. To our knowledge, this is the first report examining the impact of a *B. amyloliquefaciens* operational group strain in a distal colon model using a standardized human fecal inoculum. Phylogenetic analysis performed in this model showed that ADS024 had a selective impact on the healthy human colonic microbiota (Figures 3 and 4). This finding is similar to the *in vivo* studies performed in miniature swine (Figures 5 and 6). We consider this selective impact of ADS024 on the resident commensal microbiota an important feature for its use as a therapeutic agent to prevent rCDI. Specifically, *Bifidobacterium* species expansion in ADS024-treated samples (Figure 3C and D) was noteworthy. The correlation between *Bifidobacterium* species consumption and improved human health has been well documented[35]. Various illnesses[36,37] and aging[36-38] have been associated with reduced numbers of *Bifidobacterium* species, which, in a healthy population, positively impact the immune system[39] and are thus important for health

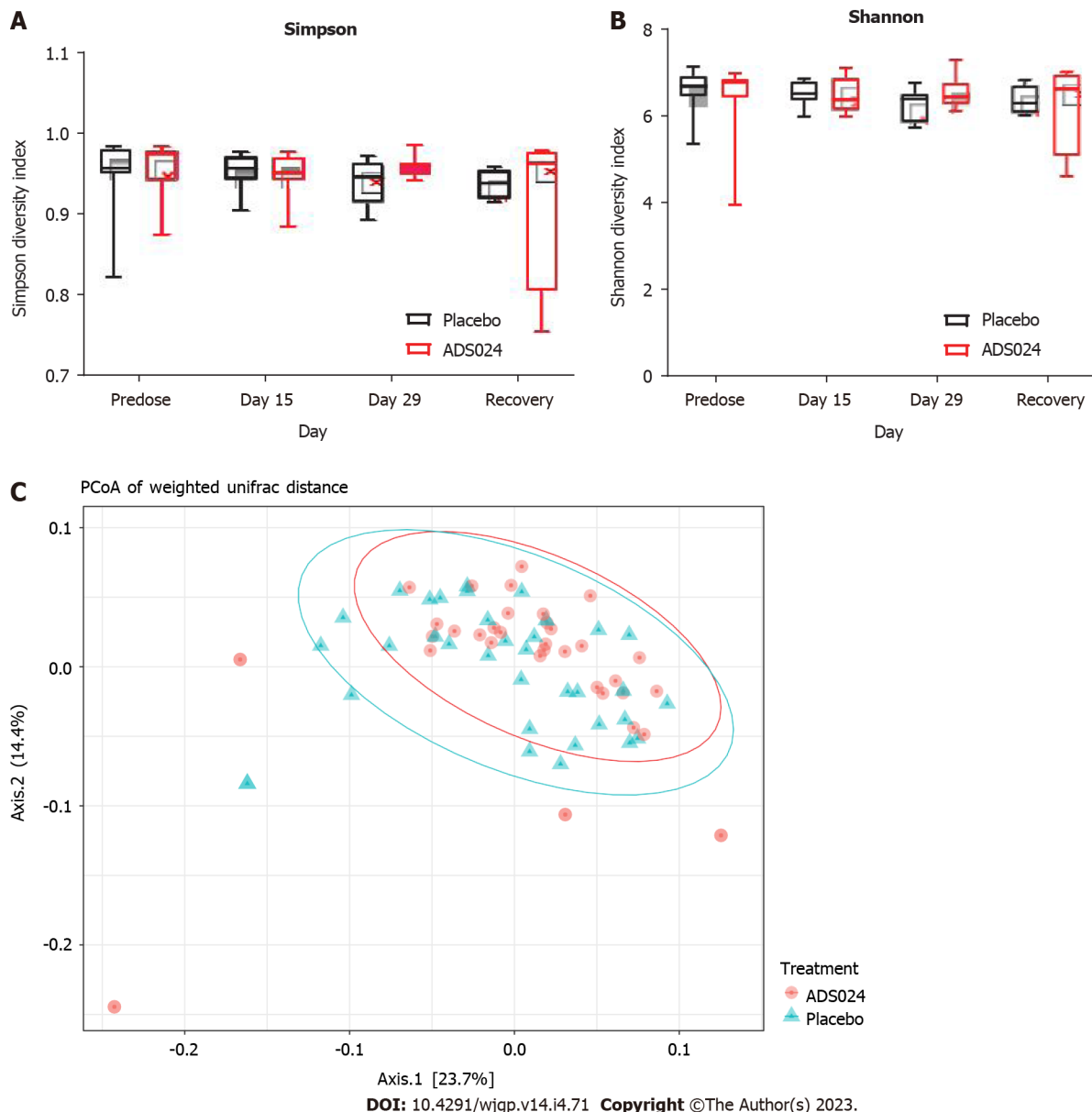


Figure 5 The impact of ADS024 on microbiota diversity in the miniature swine fecal samples during the 28-d study. MiSeq compositional sequencing and bioinformatic analysis were performed to measure the impact of ADS024 on the microbiota in miniature swine fecal samples after 28 d using the: A: Shannon index for alpha diversity; B: Simpson diversity index for alpha diversity; C: UniFrac principal coordinates analysis (PCoA) for beta diversity.

maintenance[36]. ADS024 appeared to have the added benefit of expanding this healthy bacterial population.

Recent research demonstrates the benefits of consuming *B. velezensis* isolates and their role in regulating the intestinal innate immune response and decreasing intestinal inflammation caused by pathogens in crucian carp[40]. The immunomodulatory and anti-inflammatory effects of *B. velezensis* and other related *Bacillus* species have also been studied in plants and mice[41,42], in addition to the human epithelial cells described above[31], showing their potential for treating inflammatory diseases caused by pathogens like *C. difficile*. By killing *C. difficile* and degrading its toxins[19], thereby eliminating inflammatory stimuli, ADS024 may also have offered anti-inflammatory protection, a crucial asset in tackling an inflammatory disease such as CDI.

A limitation of our studies was using the *in vitro* human distal colon model, which has limited physiological relevance. A batch fermentation system inevitably precludes the exchange of nutrients or waste removal. The accumulation of waste can potentially influence the bacterial microenvironment. Future assessments will include tissue histology and co-localization (*C. difficile*–ADS024) experiments to further characterize the mechanistic details and therapeutic effects of ADS024. Furthermore, a definitive demonstration that the direct activities of ADS024 on *C. difficile* are responsible for its biological activity in mice will require *in vivo* studies utilizing *C. difficile*–infected gnotobiotic mice.

Although safety in humans requires further study, our ADS024 findings were promising and support the continued development of ADS024 as an SS-LBP to prevent CDI recurrence following the completion of standard antibiotic treatment. In contrast to other LBPs that aim to restore diversity and prevent outgrowth by outcompeting the pathogen, we propose that ADS024 functioned by delivering bioactivities (described in O'Donnell *et al*[19,27]) directly targeting *C. difficile* and its toxins. Fecal microbiota transplantation and most LBPs may require antibiotic administration prior to

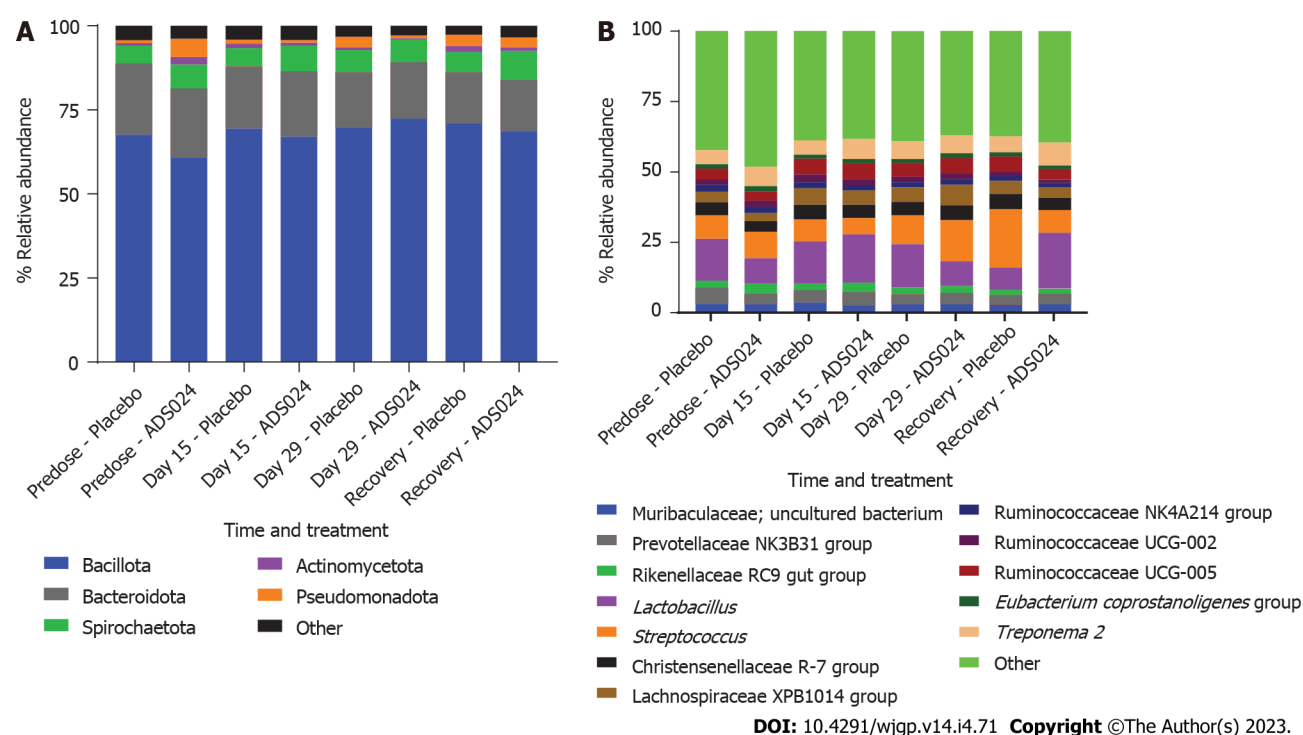


Figure 6 Effect of ADS024 on the gut microbiota of miniature swine during the 28-d study at the phylum and genus levels. A and B: MiSeq compositional sequencing and bioinformatic analysis was performed to measure the impact of ADS024 on the gut microbiota of miniature swine at the (A) phylum and (B) genus levels.

treatment to allow engraftment[43]. The lack of colonization by ADS024 contrasts with other LBPs, which depend on colonization. Given the emerging data on how microbially produced metabolites can modulate human pharmacology, the most minimally invasive, transient LBP intervention (ADS024) may be preferable. Transplanted microbiota, while effectively reducing rCDI, may cause chronic changes to the patient's natural GI microbiome as a cost of treating CDI[43, 44].

It is clear that while the burgeoning LBP field has high therapeutic potential, method optimization and the identification of selective products with fewer systemic side effects require substantially increased effort. To this end, a risk assessment should be undertaken in the early stages of LBP development[23]. The risk assessment and safety analysis of ADS024 demonstrated it to be selective, with little potential for colonizing the gut (mouse and miniature swine models), and susceptible to all the antibiotics tested for which interpretative criteria are available (Table 1). If a patient were to develop a systemic infection with ADS024 (which is not expected due to the lack of virulence factors in ADS024), a wide range of antibiotics would be available to treat the infection. *In silico* safety assessments indicated that ADS024 had a low potential for off-target activity or virulence and antibiotic-resistance mechanisms (Table 1; Supplementary Figure 1).

CONCLUSION

In vivo evaluation of ADS024 is consistent with the results of previous *in vitro* studies from our group[19,20] that showed potent *C. difficile* inhibition and maintenance of gut microbiota diversity. The totality of the evidence to date supports additional preclinical studies and early-phase clinical trials to evaluate the safety and efficacy of ADS024 in patients recovering from CDI.

ARTICLE HIGHLIGHTS

Research background

Clostridioides difficile (*C. difficile*) is a Gram positive pathogen that causes *C. difficile* infection (CDI). It infects millions of people worldwide, causing potentially life-threatening gastrointestinal disease in individuals with disrupted gut microbiomes. Following successful antibiotic treatment, recurrent CDI (rCDI) can occur in cured patients. Therefore, development of a therapeutic product for preventing rCDI following successful standard-of-care antibiotic therapy is of vital importance.

Research motivation

C. difficile, a major cause of infectious disease death in the United States, causes inflammation of the colon and potentially deadly diarrhea. ADS024, a *Bacillus velezensis* strain, has previously demonstrated direct *in vitro* bactericidal activity against *C. difficile*, without affecting other members of the gut microbiota. In this study, we investigated the efficacy of ADS024 against CDI challenge *in vivo*. Following our findings, further investigation of ADS024 as a single-strain, live biotherapeutic product (SS-LBP) for prevention of rCDI following successful standard-of-care antibiotic therapy is warranted.

Research objectives

The objectives of the research were to investigate: (1) The *in vivo* efficacy of *B. velezensis* ADS024 in protecting against CDI challenge in mouse models; (2) the capability of ADS024 to colonize the GI tract; and (3) the impact of ADS024 on the gut microbiome, finding that it was efficacious against CDI challenge without colonization, and with minimal effects on the gut microbiome, thus supporting further development of ADS024 as an SS-LBP for prevention of rCDI.

Research methods

Mouse models were used to determine: (1) The *in vivo* efficacy against CDI challenge; and (2) colonization status of ADS024 (in conjunction with miniature swine). Human distal colon model and miniature swine were utilized to determine the effects of ADS024 on the gut microbiome. To mimic disruption of the gut microbiota, the mice and miniature swine were exposed to vancomycin prior to dosing with ADS024. To model the human distal colon, an anaerobic fecal fermentation system was used.

Research results

Single oral daily doses of ADS024, similarly to multiple doses, demonstrated efficacy in protecting against subsequent challenge by *C. difficile* in a mouse model of CDI challenge. ADS024 showed no colonization based on lack of recovery ADS024 colonies in fecal samples 24 h after single doses in mice, 72 h after single doses in miniature swine, or a 28-d repeat-dose study in miniature swine. Phylogenetic analysis in the human distal colon model and *in vivo* studies performed in miniature swine demonstrated a selective impact of ADS024 on the healthy human colonic microbiota.

Research conclusions

In vivo efficacy of ADS024 in protecting against CDI challenge and minimal effects on the gut microbiome support development of ADS024 as an SS-LBP in preventing rCDI following standard antibiotic treatment.

Research perspectives

In vivo investigation of ADS024 is compatible with previous *in vitro* studies that showed efficacy against *C. difficile* and maintenance of gut microbiota diversity. Altogether, findings from these studies support initiation of clinical trials to evaluate the safety and efficacy of ADS024 in patients recovering from CDI.

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FOOTNOTES

Author contributions: Murphy CK, O'Donnell MM, Hill C, Ross RP, Rea MC, Farquhar R, and Chesnel L designed the experiments; O'Donnell MM, Hegarty JW, and Schulz S conducted the research; data was acquired and interpreted by O'Donnell MM, Hegarty JW, Schulz S, and Chesnel L; Chesnel L drafted the manuscript; all authors reviewed the manuscript and approved the final version.

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