ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2023 January 27; 14(1): 1-12



Published by Baishideng Publishing Group Inc

WJBC

World Journal of Biological Chemistry

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INDEXING/ABSTRACTING

The *WJBC* is now abstracted and indexed in PubMed, PubMed Central, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Yi-Xuan Cai*; **Production Department Director:** *Xu Guo*; **Editorial Office Director:** *Yun-Xiaojiao Wu*.

NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Vsevolod Gurevich, Jean-Marie Exbrayat, Chunpeng Craig Wan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1949-8454/editorialboard.htm

PUBLICATION DATE
January 27, 2023

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ONLINE SUBMISSION
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World J Biol Chem 2023 January 27; 14(1): 1-12

DOI: 10.4331/wjbc.v14.i1.1

ISSN 1949-8454 (online)

REVIEW

Marine biological injuries and their medical management: A narrative review

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Specialty type: Medicine, general and internal

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): E

P-Reviewer: Pitton Rissardo J, Brazil; Stachiv I, Czech Republic; Welter J, Switzerland

Received: June 27, 2022
Peer-review started: June 27, 2022
First decision: September 22, 2022
Revised: October 29, 2022
Accepted: December 13, 2022
Article in press: December 13, 2022
Published online: January 27, 2023

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Abstract

The marine environment can be extremely dangerous, and the harm caused by marine organisms when they contact the human body can be especially harmful, even deadly. Contact includes stings, bites, wounds, and consumption as food. In this article, the characteristics of the common marine biological injuries are summarized, the major marine organisms causing damage in China's marine waters are described, and injury prevention and treatment methods are discussed.

Key Words: Marine organism; Injuries and wounds; Prevention; Treatment

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Core Tip: With the changes occurring in the marine environment caused by the climate change and the continuous development of marine resources, marine biological injuries are increasing. This article classifies marine biological injuries such as stabs and bites, describes the characteristics of infections by marine microorganisms, and summarizes the prevention and treatment of major marine biological injuries in China's sea areas.

Citation: Geng XY, Wang MK, Chen JH, Xiao L, Yang JS. Marine biological injuries and their medical management: A narrative review. *World J Biol Chem* 2023; 14(1): 1-12
URL: https://www.wjgnet.com/1949-8454/full/v14/i1/1.htm
DOI: https://dx.doi.org/10.4331/wjbc.v14.i1.1

INTRODUCTION

In recent years, due to changes in the marine environment caused by the climate change, there have been outbreaks of some harmful marine organisms, such as jellyfish and microorganisms, and the rate of marine biological injury has increased yearly. Tens of thousands of people worldwide are injured every year. For example, off the coast of Australia, about 10000 people are injured by jellyfish stings every year. Marine biological injuries can be roughly divided into marine microbial infections, marine plant or animal stabs and bites, which can range from local skin damage to systemic poisoning reactions [1]. This article was written in response to the greater need for a reference on the prevention and treatment of these injuries for coastal residents, naval officers, and soldiers who may be involved in the accelerated implementation of China's marine development strategy in their daily life and training. For this article, we reviewed various academic papers written in the past 30 years since 1993, classified them according to the types of marine biological injuries mentioned, described the characteristics of the injuries and the key points of prevention and treatment, and put forward future research directions and suggestions for existing problems.

THE BASIC SITUATION OF MARINE BIOLOGICAL INJURIES RESEARCH

Using the search terms "marine biological injury" in Wanfang, China National Knowledge Infrastructure (CNKI), PubMed, and other public databases, a total of 842 research reports related to marine biological injury were found from 1993 to 2022. We found that 612 articles written in Chinese accounted for 43.3% of the total. The 801 articles in English accounted for 56.7% of the total. There were an additional 175 articles in Chinese that were also reviewed, accounting for only 12.4% of the total. After a more accurate search for marine biological injury, taking the Chinese literature found in Wanfang and CNKI as an example, there were only 52 related articles. In general, the number of cases of marine biological injury has increased since 2005, and attention has been paid to the research on marine biological injury, showing an increasing trend year by year (Table 1). The domestic research on marine biological injury and its prevention and treatment are still in development compared with foreign countries.

CLASSIFICATION AND INJURY CHARACTERISTICS OF MARINE ORGANISMS

According to the investigation by Liu *et al* in 2020[2], during the coastal training of the Chinese army, the officers and soldiers commonly encountered a variety of toxic marine organisms such as jellyfish, sea snakes, sea urchins, conches, and stinging fish. The main types of marine biological injuries were jellyfish stings and sea snake bites, of which jellyfish stings accounted for about 60.81% of the total number of those reported. This finding is roughly the same as the 2009 survey results reported by Zhong *et al*[3]. Zeng's survey showed that marine biological injuries in the 10 sea ports and sea areas of the South China Sea during 2002–2006 were mainly jellyfish stings, stingray stings, and sea snake bites. Among them, jellyfish stings accounted for 58.83% of the total number of reported injuries, stingray stings accounted for 16.41%, and sea snake bites accounted for 6.2%. Most of these injuries were associated with bacterial infections. For example, *Clostridium* spores are a common source of infection and have greatly increased the difficulty of treatment and the rate of disability and death. That said, prevention and control measures for all types of marine biological injuries can be effective, but according to the report by Liu *et al*[2], taking jellyfish stings as an example, less than 30% of subjected Chinese soldiers have correctly mastered these prevention and control measures against marine biological injuries, and this issue is likely to have a negative impact on the combat strength of Chinese officers and soldiers at sea. In the next section, we describe three main categories of marine biological injuries: microbial infection, animal stings, and animal bites.

Marine microbial infection

There are about 10 to 200 million kinds of bacteria in seawater, of which 95% are gram-negative bacteria and facultative anaerobic bacteria. The distribution of seawater samples from the East China Sea and the Yellow Sea showed that the *Vibrio* genus ranked first, accounting for 52.9% of the samples, including *V. alginolyticus*, *V. parahaemolyticus*, *V. fluvialis*, *V. vulnificus*, and *V. carchariae*. The next common source of microbial infections is Enterobacteriaceae, including *Escherichia coli* and *Enterobacter cloacae*, which mainly come from human sources and pollution[4,5]. Disease related to Enterobacteriaceae is mainly caused by food, and there are a few cases of marine Enterobacteriaceae. The *Bacillus* genus also has strong pathogenicity. These microbes are mainly introduced into the body through a wound caused by a marine organism. After a wound caused by a marine organism is soaked in seawater, the infection rate of *Bacillus* is high, about 15%. The next section will mainly summarize the research on Vibrionaceae and *Bacillus* (Table 2).

Table 1 Research and development status of marine biological injury from 1993 to 2022

The number of studies in the literature			
Published time	**Case analysis**	**Prevention and control measures**	**Review**
1993-1998	7	7	16
1999-2004	12	22	21
2005-2010	25	38	32
2011-2016	32	37	43
2017-2022	30	50	61
Total	106	154	173

Table 2 Characteristics of marine bacteria and therapeutic drugs

Bacillaceae (genus)	Bacterial species	Characteristics	Therapeutic drugs
Vibrio family	*Vibrio alginolyticus*	Strongest salt tolerance; prone be involved in infection after trauma	Imipenem, meropenem, cefepime, ceftazidime, chloramphenicol, tetracycline, levofloxacin
		Combined with seawater immersion	
	Vibrio parahaemolyticus	Mainly exists in seafood, common bacteria of food poisoning	
	Vibrio fluvialis	Mainly transmitted through the seafood, gangrene is likely to occur if the wound is infected	
	Vibrio vulnificus	Rapid onset and high mortality	
	Vibrio carchariae	Rare report	
Bacillus family	*Clostridium tetanus*	Strong vitality and can be implanted through sponge abrasion, fin stabbing, *etc.*	Prophylactic vaccination with tetanus antiserum
	Anaerobic *Clostridium perfringens*	The disease develops rapidly and can be implanted through fin stabbing, *etc.*	Prophylactic inoculation with gas gangrene antitoxin

Vibrio genus

Vibrio is widely distributed in the ocean. More than 60 kinds of *Vibrio* have been isolated from the ocean, 12 of which are related to human diseases[5-7]. The prevalence of *Vibrio* infection in humans is likely to follow regional climate trends and erupt during unusually warm weather[8]. Pathogenic *Vibrio* can cause intestinal infections, wound infections, food poisoning, and other diseases. Marine *Vibrio* has special properties including being halophilic, psychrophilic, barophilic, and oligotrophic. The secondary infection caused by marine *Vibrio* is different from an ordinary bacterial infection. *V. alginolyticus*, *V. parahaemolyticus*, *V. fluvialis*, and *V. vulnificus* are the main pathogenic Vibrio representatives in China's marine waters. They have been documented to be involved in marine environment injuries related to war, trauma, and bacterial infections[9]. *V. alginolyticus* is one of the most salt-tolerant *Vibrio* species. It is also one of the most isolated *Vibrio* species in the coastal waters of China. *V. alginolyticus* is also an important pathogen of infection after wound trauma combined with seawater immersion. It can cause acute enteritis, food poisoning, otitis media (ear infection), wound infection, bacteremia, and other diseases by producing enterotoxin, hemolysin, and other harmful substances produced by pathogens [10]. Regarding otitis media, it has been reported that the production of bacterial toxins, coupled with excessive immune response[11,12], can lead to inflammatory damage to cochlear structures and sensory cells and ultimately lead to devastating sensorineural hearing loss[13]. Unfortunately, *V. alginolyticus* infection is not easily diagnosed, but early treatment with an external antibiotic can increase the chances for rehabilitation and full recovery from hearing damage[14]. *V. parahaemolyticus* exists in seawater, fish and shrimp, shells, and processed seafood. It is a common pathogen of food poisoning in China's coastal areas for troops stationed on islands and on long-distance ships. According to statistics, this bacterium was also the main cause of bacterial infectious diarrhea in southern China from 2007 to 2012[15]. Climate warming may further cause these sickness emergencies[16]. For example, large-scale and collective food poisoning caused by this bacterium often occurs in the summer, when the weather is much warmer. The pathogenic factors of *V. parahaemolyticus* include enterotoxin, heat-resistant direct hemolysin, and similar hemolysin. In addition to food poisoning and acute diarrhea, *V. parahaemolyticus* can also cause wound infection and bacteremia[10]. Summer is the season of eating more raw and cold

food and seafood, and it is also the peak of the prevalence of intestinal infectious diseases. The clinical characteristic symptoms of diarrhea and food poisoning caused by pathogenic *Vibrio* are less severe, which makes this type of infection more easily ignored and addressed.

V. fluvialis is a pathogen transmitted by mouth. The human body is infected by ingesting polluted water and food. Common foods that are connected to this form of infection include fish, shrimp, crabs, oysters, clams, and snails. Poisoning results from, for example, ingesting raw seafood, repeated pollution after cooking has taken place, and cross-pollution between raw and cooked seafood. The sickness from *V. fluvialis* mainly causes acute gastroenteritis, and there are also reports of extraintestinal infections such as hemorrhagic cellulitis, encephalitis, bacteremia, acute otitis, and peritonitis[17]. The symptoms of gastroenteritis are very similar to cholera[18]. The typical symptoms are watery stool, abdominal pain, mild to severe dehydration, and often a fever. If a wound directly contacts the water or seafood polluted by *V. fluvialis*, the bacteria or the virulence factors produced by them will quickly fester and form gangrene, which may also cause cellulitis. An important symptom to notice for clinical diagnosis of this type of infection is hemorrhagic herpes[19,20]. *V. vulnificus* causes the most serious disease among pathogenic *Vibrio*. The course of sepsis caused by *V. vulnificus* is rapid and often fatal. Once infected with sepsis, the initial mortality rate exceeds 60%[20], the highest mortality rate among all foodborne pathogens[21,22]. Its pathogenic factors include enterotoxin, hemolysin, cytotoxin, protease [23], iron chelator[24], adhesion factor, and phospholipase A2[25], which can cause intestinal and extraintestinal infections, such as primary sepsis, traumatic infection, and acute gastroenteritis. And most of these conditions may also develop into sepsis, severe cellulitis, and necrotizing fasciitis. *V. vulnificus* infection tends to occur in men over 45-years-old or in patients with potential liver damage [21]. Infection occurs through raw seafood and open wound contact with seawater[26]. Gastrointestinal symptoms of infected patients are usually not obvious, but systemic poisoning symptoms are common, and the mortality rate is high. Therefore, it is very important to prevent *V. vulnificus* infection. Patients with chronic liver disease and kidney disease should not eat raw seafood, and people with skin wounds should avoid contact with seawater.

Bacillus genus

Bacillus can form spores (endospores), which have a strong resistance to harmful external factors and are widely distributed. The endospores have high-temperature resistance, rapid reactivation, strong secretory enzymes, and the ability to survive in both aerobic and anaerobic environments. Marine *Bacillus* is also very hardy and can form spores under unsuitable living conditions. As dormant bodies, endospores can quickly revive under suitable conditions. The common marine bacilli causing injury mainly include *C. tetanus*, anaerobic *C. perfringens*, and a genus found in marine fish, *Mycobacterium. C. tetanus* is a gram-negative bacterium, and its propagules are gram-positive. Its resistance is similar to that of other bacteria, but its spores have especially strong resistance. They can endure boiling for 40–50 min and can survive in the soil for decades. The bacterium is often implanted into the human body through a sponge abrasion or by fin stabbing by a stone fish, scorpion fish, or lionfish[27]. The incubation period is 2–21 d[28,29]. Its typical tetanus are clenched teeth, angular tension, and dysphagia. Patients often die of physical failure, asphyxia, or pulmonary complications[30].

Anaerobic *C. perfringens* is one of the most common pathogens of gas gangrene in the clinic. The name of the condition is related to the fact that the bacterium can decompose the sugar in muscle and connective tissue, produce a large amount of gas, and cause severe emphysema. The bacterium often enters the human body through an injury caused by sharp fish fins or marine animals' bones or the bite of marine animals. An infection results, and a systemic infection of *C. perfringens* may lead to the production of gases and toxins, leading to tissue necrosis[31] and gas gangrene. The incubation period is 1–2 d, and the onset is acute. The main manifestations are severe pain in the injured body part, deep mass muscle necrosis, and a purple-black coloration of the skin. There are also serious and bloody secretions flowing out of the wound. If the skin around the wound is gently pressed twists, the number of white blood cells increases, and the cardiopulmonary resuscitation value increases[32]. The patient shows obvious anemia, the body temperature and pulse speed rise, and the condition gets worse rapidly. If not treated properly, the injured often die within a few days.

Stabbing injuries caused by marine animals

Stabbing injuries caused by marine animals are probably the most common. Marine animals use their stings and thorns to commit attacks and defend themselves. The injected venom from a stinger into the human body can cause localized poisoning, skin damage, and even systemic poisoning, leading to shock and even death. This section will focus on the types of stabbing injury and related research.

Stabbing type

Regarding stabbing injuries from coelenterates such as jellyfish, anemones, and corals, the stinger bag or stinging device has an osmotic pressure of 140 atmospheres in the bag. When encountering stimulation, it can shoot the stinger violently, with a speed 40000 times the acceleration of gravity. This force can penetrate not only human skin but also crustaceans[33]. When the barb of the stinger inserts into its target, it is difficult to remove. With a light injury, there will be skin redness, swelling, heat and pain.

With a more severe injury, there may be organ failure or even death. Jellyfish stings are the most common gill stings. Jellyfish stings often occur in warmer months of the year. It is estimated that there are about 150 million jellyfish stings worldwide every year[34,35]. Jellyfish are widely distributed in the subtropical or tropical waters found on the Atlantic, Pacific, Asia, Pacific, and Australian coasts. There are a wide variety of jellyfish, and they can cause more than 100 kinds of human poisoning. Jellyfish are considered the most harmful toxic marine animal to human beings[36]. In 2022, a survey of officers and soldiers in a marine training unit showed that 136 of the 222 officers and soldiers surveyed had been injured by marine organisms during the coastal training, accounting for 61.26% of the total number surveyed. Among them, the type of injury with the highest incidence was the jellyfish sting, accounting for 60.81%. After a human is stung by a jellyfish, the victim will feel an immediate tingle followed by a feeling of burning as if hot oil has hit the skin. In the stung area of skin, rashes, blisters and bleeding spots will soon appear. Scratching aggravates the pain. In severe cases, there may be gastrointestinal dysfunction, hypotension and vomiting, massive sweating, restlessness, excessive trembling, fever, tachyarrhythmia, and hypertension[37]. Life-threatening conditions such as hypertensive cardiogenic shock, pulmonary edema, or cerebrovascular accidents may occur under severe toxicity. The lethal rate of a highly toxic jellyfish sting such as from the box jellyfish stinger is about 15%–20%, and there is a lack of specific therapeutic drugs for this type of injury.

Sea anemones are mainly distributed in the intertidal zone or hidden in reef walls or grooves and live a sedentary life. Because of their simple structure and no central information processing mechanism, they do not have the ability to attack actively. They poison mainly through skin or digestive tract contact[38]. Among the sea anemones, there is the rock sand sea anemone toxin with strong toxicity, and the lethal dose 50 by intravenous injection is 63–83 ng/kg[39]. When this toxin takes effect, the coronary artery contraction is 100 times stronger than that of angiotensin[40]. It is a typical cardiotoxin, and the strongest known coronary artery constrictor. The toxin can cause the patient to have a sudden heart attack and die within a few minutes. The local symptoms are generally edematous erythema and papules, which subside after several days. It is necessary to distinguish these symptoms from jellyfish stings. The tentacles of anemones are short, and the skin symptoms are multiple red and round spots [41]. In severe cases, local burning pain and tingling sensation occur within a few minutes, followed by blisters, bleeding, or ulcers. The systemic symptoms mainly include cardiovascular, neuromuscular, gastrointestinal and renal dysfunction, and occasionally there are cases of death[42].Corals are mainly distributed in the South China Sea. Most of them grow on reefs in clusters, forming unique corals, such as the Shizhi coral, Pufu coral, Cang coral, and so on, with a total of more than 100 species. Coral polyps are small, hydra-type individuals. They are hollow, cylindrical, and have mouths at the top. There are up to eight or more tentacles distributed around the mouth. Barbed cells are distributed on the tentacles to release barbed sacs that can paralyze prey[43]. However, these stinging cells rarely attack humans. At present, coral injuries are often caused by accidental encounters with coral (by hitting or rubbing against it) while working in the sea or swimming or snorkeling. The skin wounds usually heal slowly and tend to cause inflammation.

Ratchet type

A ratchet-type of stabbing injury is mainly caused by echinoderms, such as sea urchins, starfish, and marine vertebrates, such as stingrays and stone fishes. Echinoderms have a layer of skin with spines on their body surface. There are pedicles on the hard shells between the spines, and most of them have poison glands that can be used for defense and attack[44]. The tail spines or fin spines of the marine vertebrates mentioned above are also toxic. They not only cause wounds but alsoinject poison causing muscle spasms, nausea, vomiting,and other systematic symptoms.Sea urchins have a hard shell, and there are many thorns (spines) attached to the shell. Most of these thorns have barbs, which can increase mechanical damage to skin in the event of an injury. The neurotoxins in the thorns can poison people. There are 28 species of poisonous sea urchins in China, most of which live in tropical shallow sea reefs, especially in the Nansha Islands[44]. If you are accidentally stabbed by a sea urchin, the injury will immediately produce pain or a burning sensation, which is often accompanied by burning erythema, swelling, bleeding, and purple or black discoloration of the skin[45]. If the stabbing location is in the spine or joints, it may also cause synovitis, which lasts from 2 mo to 1 year[46]. It is reported that if multiple punctures (> 15-20 punctures) occur, systemic effects result including paralysis, bronchospasms, and hypotension, but this has not been confirmed[47].

Starfish, also known as sea pans in China, have many varieties and a wide distribution. Most of them store the toxin. They are non-toxic as long as they are not eaten. However, some starfish, such as the spiny crown starfish, have many thorns containing venom, which can also cause contact poisoning[47, 48]. These venom stings can increase the permeability of blood vessels resulting in edema, bleeding, and muscle necrosis. After stabbing by one of these spines, the injured person experiences extreme pain, redness, swelling, and numbness near the wound, accompanied by continuous vomiting and limb paralysis[33]. Stingrays, also known as devil fish and commonly known as pot fish in China because they looks like a kind of pot, have tails with poisonous spines. The main components of the venom are nucleotidase and phosphodiesterase[49]. The venom has great neurotoxicity. Fortunately, a stingray stabbing injury is relatively rare. Usually, they only attack each other with tail stabbing. However, when people press, grasp, or step on their tail, and the attack usually affects the limbs[50]. The venom enters

the body at the wound, mainly neurotoxic symptoms result. These include nausea, vomiting, muscle spasms, shortness of breath, other spasms, arrhythmia[51], and local and even systemic infection. In severe cases, the injured person may die or have multiple organ failures[52]. The effects of the injury progress rapidly, extensively involve the body, and easily lead to infection as well as secondary degeneration and necrosis of tissue cells[53]. The stonefish is one of the most toxic fish in the ocean. It mainly exists in the tropical waters of the Pacific and Indian oceans. China has reported many cases of stonefish poisoning. Toxic spines are distributed in the dorsal fin, gluteal fin, and ventral fin of the stonefish, and there are toxic sacs on both sides of the dorsal fin spines. The toxins in the toxic sacs show hyaluronidase activity, muscle toxicity, neurotoxicity, and hemolysis. These toxins can make blood vessels contract strongly, they increase blood vessel permeability[54], and they may lead to the reduction of myocardial contractility[55]. After being stabbed by the poisonous spines of a stonefish, an injured person may not feel affected instantly, but they may experience severe pain 1 hlater[56]. This pain may be accompanied by unstable blood pressure, edema, nausea, dizziness, chest tightness, delirium, other symptoms, and even death in severe cases[57].

Marine animal bites

Marine animal bites may be the most dangerous kind of marine biological injury because in addition to soft tissue laceration and hemorrhagic shock, there is also the invasion of venom, which can lead to heart failure or even death. The death rate of sea snake bites is as high as 10%, which is the most serious injury among marine organisms. All sea snakes are poisonous. However, this does not mean that all sea snake bites will release venom. About one-third to one-half of all sea snake bites will not release venom [58]. There are nine genera and 15 species of sea snakes in the southeast waters of China, mainly including the black-headed sea snake in the East China Sea and the black-tailed sea snake in the South China Sea, and the followed is the green ring sea snake, known as the “snake king,” and the green grey sea snake and the ring sea snake are common in Hainan[59]. Sea snakes are usually docile, but their aggression will increase during the mating season or when they are mistreated. For example, sea snake bites often occur when catching and killing sea snakes. At first, most people bitten by sea snakes only feel that their skin is stabbed, and there are a pair of tooth marks on their skin. There is usually no pain, itching, bleeding, or swelling in some parts. However, the venom secreted by sea snakes through venom glands eventually affects the nervous system of the human body 0.5–l h after the injury. The injured person can experience paralysis in all four limbs, drooping eyelids, difficulty speaking clearly, dysphagia, progressive dyspnea, and severe cases can lead to respiratory failure or acute renal failure [60], heart failure, asphyxia, and death. The inconspicuous nature of sea snake bite marks, the absence of local reactions, and nonspecific pathological reactions, make the diagnosis of sea snake bites difficult. Therefore, the potentially lethal nature of sea snake venom neurotoxicity makes this difficulty troublesome[61]. However, if no symptoms appear within 6–8 h, the possibility of infection can be ruled out[47].Shark bites do not often occur[62]. Generally, if people do not provoke or play with them, they rarely take the initiative to hurt people. The teeth are sharp and irregularly arranged. Therefore, after a person is bitten by a shark, the main feature is massive hemorrhage due to large-area tissue laceration, contusion or abrasion, all of which can result in hemorrhagic shock. After the injury, patients often have dizziness, tinnitus, a pale complexion, cold hands and feet, sweating, thirst, restlessness, fear or indifference, a rapid and weak pulse, and decreased blood pressure[1]. It should be noted that the shark's mouth is a breeding ground for a variety of bacteria, and these bacteria are easily transferred to the human body in the event of a shark bite. In addition, shark bites easily cause post-traumatic stress disorder in patients.Therefore, psychological intervention and antibacterial treatment should both be considered carefully when treating shark bites[63,64].

PREVENTION AND TREATMENT OF MAJOR MARINE BIOLOGICAL INJURIES ACQUIRED IN CHINA'S SEAS

Previous reports have shown that the main biologically-caused injuries in China’s sea areas include jellyfish stings, sea snake bites, spine stings from fish like stingrays, and infections of wounds bymarine bacteria.

Jellyfish sting treatment

First, quickly remove the tentacles of the coelenterate from the wound[65]. Do not scrub the skin with fresh water or cold water so as to avoid further aggravation due to the release of toxins by the stinging cells (nematocysts). It is also not advisable to use isopropanol or acetic acid for first aid as previously reported[66]. The use of isopropanol and acetic acid in first aid can promote further release of nematocysts, which may aggravate the condition[67]. The combination of seawater, 5% sodium bicarbonate, and 1%–2% lidocaine[68] can be used for treatment. This treatment promotes an analgesic effect by blocking the sodium or calcium channels of the cell membrane of the jellyfish silk sac so as to stop further stinging[69]. For skin symptoms caused by jellyfish stings, such as blisters, ecchymosis,

bleeding spots, *etc*., local alkaline drugs and topical hydrocortisone ointment or betamethasone neomycin ointment can be applied. Additionally, a small number of painkillers and steroid hormones can be taken orally to prevent inflammation. Immunomodulators such as pimecrolimus, tacrolimus, and corticosteroids can reduce the incidence of recurrent dermatitis[70]. In the case of acute pulmonary edema, renal failure, and anaphylactic shock, treatments should be according to the corresponding medical guidelines already in place for addressing these conditions.At present, the research on antidote and treatment drugs for jellyfish stings is still in the early stage. The field medical units lack targeted and standardized antidote and treatment drugs and as well as training in first-aid measures for these types of injuries. For example, naval field medical units and some residential coastal areas of China use unproven treatment methods for jellyfish stings. The prevention of jellyfish stings involves two strategies: (1) Education about marine biological injuries needs to be improved for those who live, work, and train in and near marine waters; and (2) avoidance of jellyfish as much as possible and mastering the knowledge needed to treat stings.

Sea snake bite treatment

Immediately following a sea snake bite, wash the wound with seawater. On land, wash the wound repeatedly with clean water, soapy water, and cold saline[33]. As for the treatment of terrestrial poisonous snakes, the patient should keep the affected limb fixed and in an independent posture. Under the condition that the arterial blood flow is not blocked, pressure fixation should be taken into consideration[47]. Use cloth, gauze, bandage, or rope to perform circular ligation at the proximal end of the wound to maintain the compression state. The tightness of the ligation cloth should not affect the deep arteriovenous blood flow of the limbs. The ligation should be relaxed for 1–2 min every 15–20 min. Sea snake antivenom is the most effective drug for the treatment of sea snake bites at present. Use of this antivenom is essential to prevent severe muscle toxicity[71]. The earlier the antivenom is used, the better the effect will be. In the absence of antivenom for sea snake bites, antivenom for cobras or Bungarus antivenom can be used. Sea snake venom has cross-immunity with these two venoms. In addition, to increase survival rate, a ventilator should be used to assist with breathing, and it is important to monitor electrolytes and renal function. Fluid replacement should be carried out in time. In case of myoglobinuria, sodium bicarbonate shall be used to alkalize urine, and diuretics shall be taken to maintain the water-electrolyte balance and prevent renal failure[72].The sea snakes' activity area is not large. Most of them live in offshore waters within 60 km offshore. Most of them feed on fish. Turtles often gather at the estuary of rivers with a large amount of organic matter, and this is also an area where sea snakes often hunt. Most sea snakes, which are active at night, are sensitive to lights. In addition, the behavior of sea snakes changes with the tides. As the tide rises, snakes tend to enter newly flooded areas, and with the water level rising, snakes becoming more active[73]. When human activities are carried out at a similar time, they should try to avoid the areas where sea snakes are often active.Before going to a strange marine area for activities, people should know whether there are reports of sea snakes hurting people in that area. If there is a threat of sea snakes, it is important to be aware of the types of sea snakes present and to be prepared in the event of a sea snake bite. A sea snake bite looks like a tiny puncture wound. If such a wound is found on the skin, it is necessary to know whether the patient has been in or out of the sea snake activity area in a short time. If the patient has weak muscle pain and other symptoms, an infection should be considered, and treatment should be given immediately[47]. The prevention of sea snake bites is more important than the treatment. However, as a marine biological injury with the highest mortality rate, medical workers, especially military doctors, should be aware of this type of injury.

Stingray and other stinging fish stabbing injury treatment

For stingray and other stinging fish stabbing wounds, early emergency treatment is the key to success. The wound must be cleaned with flowing water immediately after the injury, and the blood must be extruded from the centrifugation direction near the heart to prevent the absorption of poisons[74]. Stingray venom contains a kind of hyaluronidase, which can decompose tissues and stimulate nerves causing severe pain. It is recommended to immediately immerse the wound in tolerable hot water (upper limit 45°C, 30–90 min) after washing with flowing water. In fact, a meta-analysis of hot water therapy for all stinging marine organisms, including four randomized controls and six case studies, showed that soaking at 42 °C–45 °C for 30–90 min or until the pain disappears is safe and effective for all marine stinging fish injuries[75]. If necessary, taking analgesics is recommended to relieve pain and poisoning symptoms. Stingrays and other stinging fish wounds can be treated with systemic antibiotics for 5 d at the initial stage of infection. Ciprofloxacin, trimethoprim, and sulfamethoxazole seem to be the most suitable antibiotics for the prevention or treatment of local infection[76]. Debridement and local antibacterial operations are performed on the wound, and the exudate is absorbed to lockout bacteria and water in order to prevent infection. If possible, infrared and red light treatment is useful for wound treatment (for 10-min periods). The light promotes cell proliferation and anabolism, improves microcirculation, promotes the growth of granulation tissue, and enhances leukocyte phagocytosis, which helps reduce swelling and inflammation of the wound[77].Treating stingray and other stinging fish stabbing wounds quickly is very necessary. Clean the wound with clean water immediately after the injury, tie a bandage 20 cm above the wound, and squeeze blood from the centrifugation direction near the heart is

an effective measures to prevent the absorption of poisons, and then immediately send the patient to a hospital. All stingrays and other stinging fish stabbing wounds need to be inoculated with tetanus antitoxin serum[78]. People working at sea should avoid touching or catching any animal that has spines on their backs and tails.

Treatment of wounds complicated by marine bacterial infection

The problems associated with a marine bacterial infection in a wound that took place in a marine environment followed by seawater immersion has attracted more and more attention in the medical community. Because of the different types of seawater bacteria, the infection time is often advanced, and the degree of the infection is aggravated. We cannot only rely on experience with treating microbial infections on land. Treating marine bacterial infections require different strategies. Targeted antibiotic treatment is an important treatment for wounds complicated by a marine bacterial infection. The results of bacteria isolated from seawater in China showed that the main bacteria in seawater were from the genus *Vibrio* and the Family Enterobacteriaceae. The results of the drug sensitivity test showed that *Vibrio* and Enterobacteriaceae in seawater were highly sensitive to imipenem, meropenem, cefepime, ceftazidime, and levofloxacin, with a sensitivity rate of more than 90%[5]. These antibiotics should be the first choice in the early treatment of marine operation injuries and accidental injuries that have also been affected by sea water immersion. The sensitivity to tobramycin, cefuroxime, cefazolin, and ampicillin was low[79], so these antibiotics should be used with caution or not used at all. Other antibacterial drugs should be used as alternative to these.

Marine *Vibrio* and bacilli rarely cause death after infection. However, if the infected patient has cirrhosis, diabetes, chronic kidney disease, acquired immune deficiency syndrome,low immune function, or long-term alcoholism, this individual is high-risk and the chances of mortality after infection is very high[80]. Therefore, the above-mentioned high-risk groups should avoid working offshore and/or avoid becoming injured in offshore locations. *C. tetanus* and anaerobic *C. perfringens* implanted into the human body due to sponge abrasion or stabbing injury by a marine animal cannot be ignored. If not treated properly, the mortality rate will be very high. Serum therapy should be selected for a *C. tetanus* infection, as well as intravenous infusion of tetanus antiserum 20×10^4 μL, once a day, for 2consecutive days. Additionally, intramuscular injection of chlorpromazine or intravenous drip of thiopental sodium should be used to alleviate muscle spasms. Medical caregivers should pay attention to maintaining oxygen supply and the acid-base balance in the body, and if necessary, continuous renal replacement therapy can be used[28]. Coastal residents as well as officers and soldiers stationed at sea who are stationed on long-term offshore operations can be vaccinated with Tetanus Toxoid to prevent tetanus. Anaerobic *C. perfringens* infection can easily cause gas gangrene[81]. After infection, the necrotic tissue must be removed immediately, the wound washed with 3% hydrogen peroxide and 1/5000 potassium permanganate solution, and the gas gangrene antitoxin injected into the muscle. The patient should be given high protein, high nutrition, and vitamin support to improve their resistance. Prevention of anaerobic *C. perfringens* infection mainly involves debridement as soon as possible in case of deep wounds that might lead to an anaerobic infection. Foreign bodies should be removed and the patient should be treated with a preventive intramuscular injection of dry refined multivalent gas gangrene antitoxin and antibiotic interventions such as the combination of penicillin and clindamycin [82].

CONCLUSION

Marine biological injury is a major obstacle to the improvement of China's maritime power. However, China's current research on marine biological injury is still relatively lagging behind. The United States has published a manual and first aid guide for toxic and harmful marine organisms, and asked Navy divers and Marines to master the treatment methods for toxic and harmful marine biological trauma and poisoning. Australia has developed antivenoms for organisms such as sea snakes and jellyfish, and shark repellents. By contrast, China still has a long way to go in the field of marine biological injury prevention and control. To meet the actual needs of naval forces and coastal residents, China's relevant scientific research and health personnel should focus on the living habits of common toxic marine organisms along the coast of China; the physical and chemical properties and physiological activities of related toxins; and the prevention, first aid, and treatment of common marine biological injuries and implement relevant measures; strengthen the understanding and prevention awareness of coastal residents and officers and men working at sea on marine organisms; and promote the mastery of basic first aid and prevention measures. At the same time, as the research on drugs for the prevention and treatment of marine biological injuries in China is still in the early stage, average medical units lack targeted and standardized prevention and treatment drugs and first aid measures to treat marine biological injuries. Additionally, the prevention methods and treatment methods for marine biological injuries are still relatively unscientific, uninformed, and lack effectiveness. At present, the treatment measures used are mainly only treat symptoms. Based on this status, researchers with expertise in this subject in China should speed up research in this area to achieve breakthroughs and overcome technical

difficulties. This work and research is also of great practical significance to improve the combat effectiveness of our Navy.

ACKNOWLEDGEMENTS

We thank all colleagues, the reviewers and the editors for improving our paper.

FOOTNOTES

Author contributions: Wang MK, Xiao L, and Yang JS conceptualized the article, revised the manuscript, and contributed equally to this work; Geng XY wrote the draft; Chen JH collected the literature; All authors have read and approved the final manuscript.

Supported by Scientific Research Project of Shanghai Municipal Health Commission, No. 202140061.

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

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S-Editor: Xing YX
L-Editor: Filipodia
P-Editor: Xing YX

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ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2023 March 27; 14(2): 13-61



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World Journal of Biological Chemistry

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World Journal of Biological Chemistry

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INDEXING/ABSTRACTING

The *WJBC* is now abstracted and indexed in PubMed, PubMed Central, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Yi-Xuan Cai*; **Production Department Director:** *Xu Guo*; **Editorial Office Director:** *Yun-Xiaojiao Wu*.

NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Vsevolod Gurevich, Jean-Marie Exbrayat, Chunpeng Craig Wan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1949-8454/editorialboard.htm

PUBLICATION DATE
March 27, 2023

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World J Biol Chem 2023 March 27; 14(2): 13-27

DOI: 10.4331/wjbc.v14.i2.13

ISSN 1949-8454 (online)

REVIEW

Molecular genetics of early-onset colorectal cancer

Olivia Marx, Marc Mankarious, Gregory Yochum

Specialty type: Biochemistry and molecular biology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Han J, China; Zheng T, China;

Received: November 21, 2022
Peer-review started: November 21, 2022
First decision: December 13, 2022
Revised: December 20, 2022
Accepted: February 13, 2023
Article in press: February 13, 2023
Published online: March 27, 2023

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Abstract

Early-onset colorectal cancer (EOCRC) has been rising in global prevalence and incidence over the past several decades. Environmental influences, including generational lifestyle changes and rising obesity, contribute to these increased rates. While the rise in EOCRC is best documented in western countries, it is seen throughout the world, although EOCRC may have distinct genetic mutations in patients of different ethnic backgrounds. Pathological and molecular characterizations show that EOCRC has a distinct presentation compared with later-onset colorectal cancer (LOCRC). Recent studies have identified DNA, RNA, and protein-level alterations unique to EOCRC, revealing much-needed biomarkers and potential novel therapeutic targets. Many molecular EOCRC studies have been performed with Caucasian and Asian EOCRC cohorts, however, studies of other ethnic backgrounds are limited. In addition, certain molecular characterizations that have been conducted for LOCRC have not yet been repeated in EOCRC, including high-throughput analyses of histone modifications, mRNA splicing, and proteomics on large cohorts. We propose that the complex relationship between cancer and aging should be considered when studying the molecular underpinnings of EOCRC. In this review, we summarize current EOCRC literature, focusing on sporadic molecular alterations in tumors, and their clinical implications. We conclude by discussing current challenges and future directions of EOCRC research efforts.

Key Words: Early-onset colorectal cancer; Later-onset colorectal cancer; Mutations; oncogenes; Molecular characteristics; Transcriptomics

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Core Tip: Early-onset colorectal cancer (EOCRC) has a considerably different clinical presentation and genetic profile compared with later-onset colorectal cancer. Furthermore, molecular alterations in EOCRC tumors differ in patients from separate geographical locations and distinct ethnic groups. Small human cohorts and the lack of a suitable mouse model system limit EOCRC studies, however, several actionable clinical targets and biomarkers specific to EOCRC have been identified. In this review, we discuss molecular alterations in EOCRC tumors at the DNA, RNA, and protein levels, and suggest future work to examine how these changes contribute to EOCRC pathogenesis.

Citation: Marx O, Mankarious M, Yochum G. Molecular genetics of early-onset colorectal cancer. *World J Biol Chem* 2023; 14(2): 13-27
URL: https://www.wjgnet.com/1949-8454/full/v14/i2/13.htm
DOI: https://dx.doi.org/10.4331/wjbc.v14.i2.13

INTRODUCTION

Early-onset colorectal cancer is a growing global issue

Cancers of the colon and rectum are the third most commonly found in both men and women globally [1]. Colon cancer screenings have increased early detection in patients over the age of 50-year-old and have contributed to the overall decline in global rates of colorectal cancer (CRC)[1]. However, the population of early-onset CRC (EOCRC) patients, under 50-year-old, has been steadily rising over the past several decades[2,3], and by 2030, the rates of early-onset colon and rectal cancers are expected to increase by 27.7%, and 46.0%, respectively[4]. Unfortunately, 10%-15% of CRC patients are diagnosed before the age of average-risk screening recommendation (before 2018, 50-year-old)[5]. Due to a lack of screenings and a delay in the diagnosis of younger patients, EOCRC is often detected at advanced stages, reducing the chances of long-term survival[6]. Many studies have shown that EOCRC is molecularly distinct from later-onset CRC (LOCRC), or CRC diagnosed after the age of 50-year-old. Compared with LOCRC, EOCRC has a differing frequency of oncogenic mutations[7], increased prevalence of mucinous and signet (poorly differentiated) histology[8], a more distal location[2], and exhibits a distinct DNA methylation profile[9]. Despite aggressive treatment of EOCRC patients, their overall survival is worse compared to those with LOCRC[5,10].

EOCRC risk factors

There is no clear cause for most EOCRC cases, although environmental risk factors are likely key contributors to cancer development. Lifestyle factors such as smoking, unhealthy diet, obesity, and alcohol consumption increase the risk of developing CRC early on[1]. In the United States, EOCRC has a strong birth cohort effect, implicating generational lifestyle changes in the development of EOCRC[11].

Several recent studies have demonstrated the association between obesity and metabolic disorders with the development of EOCRC[12,13]. Tang *et al*[14] found that EOCRC patients had a worse metabolic profile, with higher levels of triglycerides and lower levels of high-density lipoprotein cholesterol compared with LOCRC patients[14]. Molecular links between obesity, metabolic disorders, and CRC have been suggested, including the promotion of intestinal stem cell populations[15,16], increased insulin resistance, adipocyte levels, and inflammation[17]. How EOCRC risk factors affect clinical presentation is still under investigation. One aspect of EOCRC clinical presentation of particular interest is tumor location[18].

Differences in left- and right-sided EOCRC

Over half of pre-malignant polyps in EOCRC are found in the distal colon and rectum[18], and this has prompted calls for screening sigmoidoscopy at an earlier age than current guidelines, which were recently changed from 50 to 45[19]. While left-sided colon cancer is more predominant in EOCRC, right-sided EOCRC is associated with lower overall survival compared to left-sided EOCRC (44% *vs* 61%) [20]. Several factors have been implicated in the difference in survival between right-sided and left-sided CRC. During embryonic development, the proximal colon originates from the midgut while the distal colon originates from the hindgut. This developmental difference may impact cancer cell origins as well as the metastatic potential of tumors due to differences in vascularization. Additionally, several microbiota changes have been characterized between the proximal and distal colon which may play a role in oncogenesis[21,22]. Proximal colonic tumors also have distinct histopathological features as they tend to be more mucinous with microsatellite instability and mismatch repair (MMR) deficiency compared to distal tumors[23,24]. These distinctions may indicate unique molecular drivers of distal and proximal EOCRC tumors.

Common molecular drivers of colorectal adenocarcinoma

The multi-step progression from normal colonic mucosa to adenoma to CRC was first described in 1990 by Fearon and Vogelstein[25]. In this model, an *APC*-inactivating mutation is an initiating event, followed by *KRAS*-activating mutations driving adenoma development[25]. Further studies found that the malignant transformation of adenomas was driven by additional mutations in the tumor growth factor beta, *PIK3CA*, and *TP53* pathways (Figure 1)[26-28]. Sessile serrated polyps act as a precursor to up to one-third of CRCs and are thought to arise through mechanisms distinct from canonical *APC* mutation-driven polyps[29-31]. Instead, serrated polyps are thought to develop from a *BRAF* mutation and are also often characterized by DNA hypermethylation[29]. Mutations to *BRAF*, *KRAS*, and *TP53* are also found in other cancers and the multi-step progression to adenocarcinoma may not strictly follow the canonical or serrated pathways described. While most sporadic LOCRCs can be categorized by deregulation of canonical Wnt/β-catenin/*APC* signaling or serrated *BRAF* mutation pathways[30] (Figure 1), it is less clear how sporadic EOCRCs develop.

CRC is often categorized into consensus molecular subtypes (CMS). At the genomic level, CRC can be categorized as microsatellite instable (MSI, often caused by a defect in MMR genes) or chromosome instable. At the transcriptomic level, there are four main CMS. CMS1 is associated with immune JAK-STAT activation, microsatellite instability, and hypermutated tumor DNA[32]. CMS2 is associated with canonical Wnt/*MYC* activation, CMS3 is characterized by metabolic alterations, and CMS4 is associated with epithelial-mesenchymal transition and immunosuppression[32]. CMS1 tumors are more often considered CpG island methylator phenotype-high (CIMP-high, characterized by genome-wide hypermethylation). These CMS1 tumors also often have *BRAF(V600E)* mutations and are associated with sessile serrated adenomas[32]. *RSPO* fusions and *RNF43* mutations are often seen in hypermutated CMS1 CRC[32], and Yan *et al*[33] also identified these alterations in a subset of EOCRC tumoroids[33].

Differing frequencies of CMS in EOCRCs compared with LOCRCs have been identified. Willauer *et al*[34] showed that patients under 40 were more likely to exhibit CMS1 or CMS2, with CMS1 being considerably more prevalent in EOCRC compared with the average of most CRCs, though no differentiation between sporadic and hereditary mutations was made[34]. Therefore, the increased prevalence of Lynch syndrome, a hereditary MMR deficiency syndrome, in younger patients[7,35] could contribute to this observation. In fact, MSI/CIMP-high tumors were associated with Lynch syndrome in young patients, whereas in older patients, they were associated with *BRAF* mutations[36]. Sporadic EOCRC patients are less likely to have Lynch syndrome[37] and are less likely to have tumors with a CpG island methylator phenotype[38], which are associated with CMS1[38]. Limitations to sample size necessitate future studies to examine the CMS of sporadic EOCRCs compared with LOCRCs.

Many excellent reviews explain the molecular subtypes and mutations in CRC[30,32,39]. Likewise, many excellent reviews have focused on the different clinical presentations and outcomes of EOCRC and LOCRC patients, with some mention of prevalent molecular distinctions of EOCRC[2,4,40]. However, reviews that focus primarily on the molecular characterizations of EOCRCs are limited. There has been a rise in EOCRC literature over the past 5 years that we aimed to summarize here. In this review, we searched the literature for early- and young-onset CRC, along with specific searches for molecular genetics, DNA methylation, histone alterations, transcriptomics, splicing, proteomics, ethnic disparities, and biomarkers. Relevant papers were selected for discussion. Here, we summarize key findings of the molecular genetic underpinnings of EOCRC thus far.

GENETIC STUDIES OF EOCRC

DNA mutations associated with EOCRC

Hereditary mutations often lead to CRC at a younger age in comparison with sporadic mutations. Compared with LOCRC, EOCRC patients have an increased polygenic risk score based on profiling single nucleotide polymorphisms[41]. Although approximately 30% of EOCRC cases report a family history of related cancers, only an estimated 10%-20% have known genetic risk factors like familial adenomatous polyposis, Lynch syndrome, or inflammatory bowel disease[7,10,42,43]. Therefore, our understanding of the genetic and molecular pathways that drive carcinogenesis in most patients is far from complete.

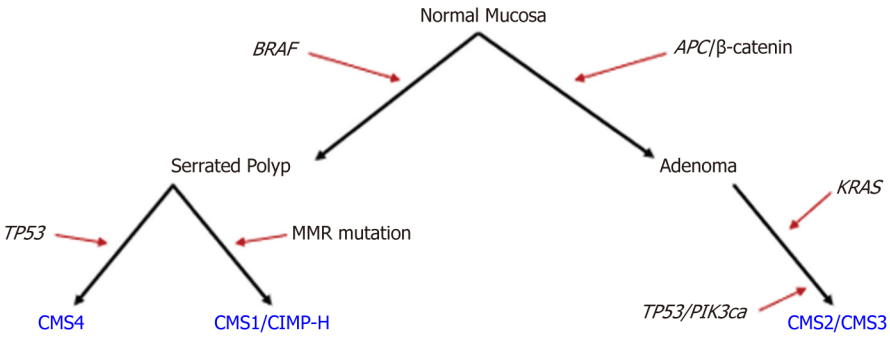
Sporadic EOCRCs, patients with no family history, have been shown to have different mutational profiles compared with LOCRCs (Table 1). Notably, many studies have found a significant decrease in the prevalence of *APC* and Wnt pathway mutations in EOCRC compared with LOCRC[33,43-45], with the exception of the β-catenin gene, *CTNNB1*, which is mutated in more EOCRCs compared with LOCRCs (Figure 2)[33,34]. Interestingly, a recent study by Yan *et al*[33] used organoids to demonstrate the heterogeneity between EOCRC patients, identifying some with *APC* mutations and others with *RSPO* fusions, which render the cultures hypersensitive to Wnt withdrawal[33].

MYC is a key oncogenic target of the Wnt/β-catenin pathway that is often deregulated in CRC[46]. Copy number variations of *MYC* are seen in 8%-15% of CRCs[47-49], however, we recently reported that 35% of EOCRC tumors from a 21-patient cohort had an increase in *MYC* copy number[50]. In addition, Pan *et al*[51] reported increased *MYC* copy number in younger CRC patients[51], however, another

Table 1 Differences in early-onset colorectal cancer and later-onset colorectal cancer DNA mutations

Gene	Prevalence in EOCRC *vs* LOCRC[1]	Role in cancer
APC	Decreased[33,34,44,45]	Blocks β-catenin, tumor suppressor
CTNNB1	Increased[33,34]	β-catenin, potentiates Wnt signaling, proliferation, and stemness
RNF43	Increased[43]/NS[33]	E3 ligase, negative regulator of Wnt signaling
BRCA2	Increased[54]	Double stranded DNA repair, tumor suppressor
PHLPP1	Increased[54]	Promotes apoptosis, inhibits *AKT*
TOPORS	Increased[54]	Regulates *TP53* stability, likely tumor suppressor
ATR	Increased[54]	*PI3/PI4* kinase, activates checkpoint proteins
MYCBP2	Increased[54]	*MYC* binding protein, activates *MYC*
FBXW7	Increased[44,54]	Ubiquitin ligase component, ubiquitinates *MYC*
POLE	Increased[44,54]	DNA polymerase E subunit, proofreading and DNA repair
BRAF	Decreased[34,38,57]/increased[43]	Proto-oncogene, activates *MAPK* signaling
TP53	Decreased[38,52]	Cell cycle inhibitor, tumor suppressor
NOMO1	Increased[53,126]	Inhibits nodal signaling. Deletion increases CRC cell migration
MYC	Increased[50,51]/NS[43,47]	Proto-oncogenic transcription factor, promotes proliferation and stemness
DNMT3B	Decreased[43]	De-novo DNA methyltransferase
MET	Decreased[43]	Proto-oncogene, promotes cell growth and survival
PTEN	Increased[57]	Tumor suppressor, negatively regulates *AKT* signaling
KRAS	Decreased[99,120]	Proto-oncogene, activates oncogenic signaling pathways

[1]Increase or decrease in the prevalence of genomic mutations or copy number variations in early-onset colorectal cancer compared with later-onset colorectal cancer tumors.
CRC: Colorectal cancer; NS: Not significant.

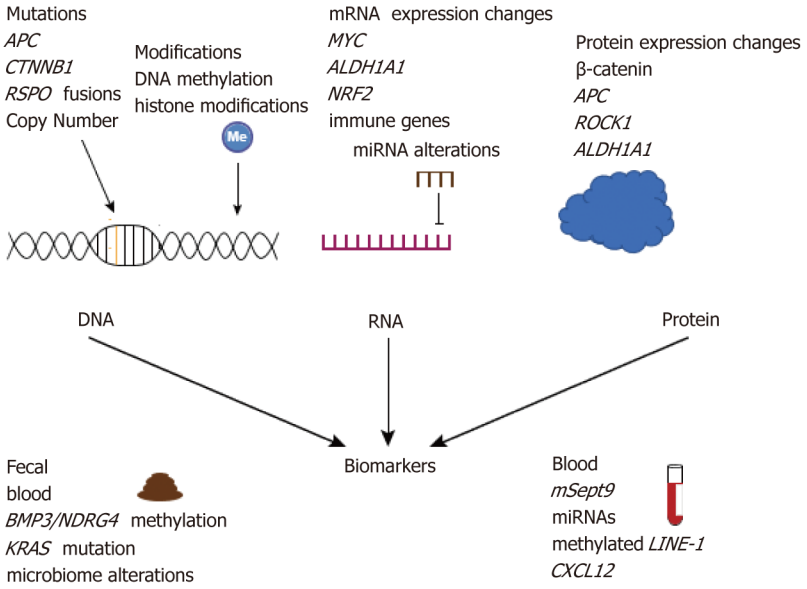


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Figure 1 Progression of normal mucosa to colorectal cancer subtypes. Shown are the major mutations in genes or pathways that have been implicated in the change from a normal colonic mucosa to cancer. In blue are the consensus molecular subtypes of cancers that arise from the preceding mutations based on transcriptomic analyses of colorectal cancer. This flow chart was assembled and modified from figures and information published in Langner *et al*[29] and Nguyen *et al*[31]. CMS: Consensus molecular subtype; MMR: Mismatch repair; CIMP: CpG island methylator phenotype-high.

study found no association between *MYC* copy number and age[47]. Overall, chromosomal deletions and copy number variations have been shown to be more common in EOCRC tumors compared with LOCRC tumors[52,53]. Alterations to *MYC* regulatory genes have also been identified in EOCRC, including *MYCBP2*[54], an E3 ubiquitin ligase that may regulate *MYC* transcription[55], and *FBXW7*[44,54], a tumor suppressive ubiquitin ligase that mediates degradation of *MYC*, among other oncoproteins, though more work is needed to determine the functional effect of these mutations[56]. Together, these findings provide evidence for a Wnt-independent increase in *MYC* activity in EOCRC.

In addition to differences in the prevalence of Wnt pathway/*MYC* mutations, many studies reported a decrease in *BRAF (V600E)* mutations (Table 1)[34,38,57], although Xu *et al*[43] noted an increase in *BRAF (V600E)* in EOCRC compared with LOCRC tumors[43]. This finding has clinical implications, as



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Figure 2 Summary of key DNA, RNA, and protein alterations identified in early-onset colorectal cancer. Shown are DNA mutations and modifications, mRNA expression changes, and protein expression changes that have been reported to contribute to early-onset colorectal cancer and that may serve as biomarkers. miRNA: microRNAs.

BRAF (V600E) mutant tumors are associated with CIMP-high status, have a worse prognosis, and respond differently to cancer treatments[58]. The decrease in *APC* and *BRAF* mutations in EOCRC indicates that a higher percentage of EOCRC tumors may not follow the canonical or serrated carcinogenesis pathways commonly observed in LOCRC (Figure 1)[34]. Overall, many key oncogenes and tumor suppressors are differentially mutated in EOCRC compared with LOCRC, which may impact cancer progression and prognosis (Table 1, Figure 1). As new mutations unique to EOCRC are uncovered, more work is needed to determine how such mutations affect gene activity.

Epigenetic modifications in EOCRC

In addition to DNA mutations, studies have suggested that EOCRC has a distinct DNA methylation profile from LOCRC[9,59]. DNA methylation regulates gene expression and has been implicated in CRC [60]. Methylation of the long non-coding RNA *LINE-1* is often thought to represent global DNA methylation[61]. Studies suggest that DNA is overall hypomethylated in EOCRC compared with intermediate or LOCRC[42,59]. Epigenetic modifications may also be detectable in the blood, and a study by Walters *et al*[62] found hypermethylation of DNA repetitive elements, including *LINE-1*, in white blood cells from EOCRC patients[62]. While previous studies examined global DNA methylation, a recent high-throughput study by Joo *et al*[9] identified 234 differentially methylated regions unique to EOCRC tumors[9]. The authors then compared EOCRC DNA methylation patterns to those which occur upon age-related methylomic drift in the normal mucosa. They suggest that EOCRC tumors more rapidly accumulate cancer-related methylomic drift compared to intermediate or LOCRC tumors, though it remains unclear when this drift occurs during cancer progression[9]. More work is needed to assess DNA methylation over time and within patient-matched tumors and normal mucosa to better understand how age-related DNA methylation changes contribute to EOCRC.

In addition to DNA methylation, histone methylation and acetylation are associated with both aging and CRC[60], however, there have been few studies on histone modifications in EOCRC. A study in 2015 found that high levels of *H3K27me3* were associated with a better prognosis in younger CRC patients and a worse prognosis in older CRC patients[63]. DNA and histone modifications are a natural part of aging, however, how they impact gene expression, cancer progression, and drug response remains to be elucidated.

EOCRC transcriptomics

Transcriptome analysis is a comprehensive tool to identify deregulated signaling pathways in cancer[64, 65]. When applied to human tissues, this approach considers both genetic and environmental factors that contribute to the profile of deregulated gene expression on a per-patient basis. Several studies have analyzed the transcriptomic profile of EOCRC; however, many studies are limited by sample size and availability of patient-matched control samples.

Deregulation of mRNA targets of the Wnt/β-catenin pathway has been demonstrated in EOCRC, though at a lower frequency compared with LOCRC[33]. We have recently published transcriptome

analyses implicating deregulated *MYC*, and its downstream targets, in EOCRC[50]. The proto-oncogene *MYC* is upregulated in the intestines of obese individuals[66] and has been suggested to control obesity-mediated metabolic dysfunction in the intestines[67], though Ellegaard *et al*[68] found no relationship between *MYC* expression and body mass index[68]. Interestingly, our recent transcriptomic study found increased *MYC* expression in the EOCRC tumors of a subset of patients who were obese, suggesting a distinct tumor gene expression profile in obese and non-obese patients[50]. We did not find significant deregulation of the Wnt/β-catenin hallmarks of cancer in our EOCRC tumors compared with adjacent normal tissue. The age-associated role of *MYC* in CRC is supported by another recent study that implicated overexpression of *MYC*, along with the lncRNA *WiNTRLINC1* and the gene *ASCL2*, in younger colon cancer patients[69].

In addition to *MYC*, studies comparing EOCRC and LOCRC have found enrichment of cell signaling, apoptosis/inflammation, proliferation, adhesion, and development[38,70]. The recent success in cancer immunotherapies has prompted an interest in examining the immune profiles of CRC[71], however, few studies have interrogated the immune response in EOCRC. A recent study highlights the importance of aging and tumor immune response, showing that aging-related gene ontology sets were enriched in CRC tissues compared with normal tissues and this signature was higher in tumors with high immune infiltration[72]. Profiling approximately 40 tumors from both late- and early-onset CRC patients, Gardner *et al*[73] found that three immune genes *SAA1*, *C7*, and *CFD*, have deregulated expression in EOCRC primary tumors compared with LOCRC tumors[73]. Changes in the expression of these genes were shown to alter the tumor immune microenvironment and are associated with intestinal inflammation[73]. Another study identified age-associated changes in tumors compared with normal tissues and found enrichment of the nuclear factor erythroid 2-like 2 oxidative stress response in the tumors of younger patients compared with older patients (Figure 2)[74]. The tumor immune microenvironment is a complex system that involves many different cell types. Immune studies in EOCRC are limited by using a homogenized tumor population for bulk RNA sequencing instead of examining alterations at a single cell level.

Most transcriptomic studies of EOCRC have focused on mRNA, however, there is increasing evidence for the relevance of microRNAs (miRNAs) in cancer. miRNAs are short RNA transcripts that generally function to bind and repress a specific target mRNA. Two notable miRNA studies have been performed for EOCRC, the first by Nakamura *et al*[75] examined miRNAs from tumors and normal samples and found a seven-miRNA panel that was upregulated in EOCRC (n = 42), but not LOCRC (n = 370), in tumor *vs* normal tissues (Table 2)[75]. An earlier study using microarray analyses of a Turkish EOCRC cohort identified downregulation of *miR-143*, *miR-125b,* and upregulation of *miR-106a* in tumors *vs* normal tissues, although no comparison with LOCRC was performed[76]. While these miRNAs have been suggested as biomarkers for EOCRC, limited sample sizes, lack of patient-matched controls, and a lack of functional studies leave their roles in cancer progression unclear.

As with most EOCRC studies, transcriptomic analyses of EOCRC are limited by sample size and availability of quality sequencing data from tumors and patient-matched normal control samples. In addition to changes in transcript abundance, RNA sequencing can provide information about alternative polyadenylation and splicing events, which can alter protein structure and function. Alternative polyadenylation is associated with cellular proliferation and cancer[77]. It serves to alter the *3'UTR* length, which can affect miRNA regulation in many cancers including CRC[78]. Unfortunately, to our knowledge, there have been no studies on alternative splicing or polyadenylation events in EOCRC. However, one study did find a *POLE* mutation that may be associated with aberrant splicing in EOCRC [79]. As aberrant alternative splicing has been implicated in both CRC[80] and aging[81], we propose that examining EOCRC-specific splicing events would uncover novel insight into disease pathogenesis. Additional post-transcriptional modifications to mRNA, lncRNA, tRNA, and rRNA, such as methylation, have been associated with CRC but remain unexplored in EOCRC[82,83].

In addition to post-translational modifications and miRNA analysis, another type of transcriptomic analysis that is gaining popularity is single-cell RNA-sequencing (scRNA-seq), which can be used to identify gene regulation in the different cell types involved in cancer. Single-cell transcriptomics has been used to analyze the age-associated transcriptome in cancers including CRC[84]. Saul and Kosinsky [84] found that many aging- and senescence-associated genes were generally upregulated in cancers, including CRC[84]. These authors also found that CRC displayed distinct populations of epithelial cells with elevated age-related gene expression, underscoring the importance of examining age-related differences in CRC at a single-cell level[84]. Understanding immune infiltration and stem cell populations is crucial for developing cancer treatments that reduce the risk of relapse. Yan *et al*[33] performed scRNA-seq of EOCRC organoids and showed differing stem cell populations in response to Wnt media supplementation for six different EOCRC and LOCRC tumoroids with different underlying mutations[33]. Expanding scRNA-seq of EOCRCs would further elucidate information about disease progression that is specific to distinct cell populations in younger patients.

EOCRC proteomics

Proteomic studies have advanced cancer treatments by identifying therapeutic targets[85]. While the proteomic signature of CRC has been established[85,86], few studies have profiled the proteome of EOCRC[74,87]. Gong *et al*[87] recently published a paper using mass spectrometry to identify age-

Table 2 Differentially expressed transcripts in early-onset colorectal cancer

Gene(s)	Description
MYC	Proto-oncogenic transcription factor, increased in EOCRC tumors *vs* normal samples[50,69]
ASCL2	Transcription factor that promotes intestinal stem cells, increased expression in younger CRC[69]
ALDH1A1	Protein involved in cancer cell stemness, expressed higher in EOCRC tumors[88]
PEG10	Promotes proliferation and invasion, increased in EOCRC tumor *vs* normal and EOCRC *vs* LOCRC[70]
miR-143, miR-125b	miRNAs, under-expressed in EOCRC tumor *vs* normal[76]
miR-106a	miRNA, overexpressed in EOCRC tumor *vs* normal[76]
hsa-miR-4304, hsa-miR-513a-5p, hsa-miR-628-3p, hsa-miR-194-3p, hsa-miR-193a-5p, hsa-miR-210, and hsa-miR-4453	miRNAs uniquely overexpressed in EOCRC compared with LOCRC and normal tissues[75]
SAA1, C7, CFD	Immune genes differentially expressed in EOCRC *vs* LOCRC tumors[73]
NRF2	Protein involved in oxidative stress and inflammation, expressed higher in EOCRC *vs* LOCRC[74]

CRC: Colorectal cancer; EOCRC: Early-onset colorectal cancer; LOCRC: Later-onset colorectal cancer; miRNA: microRNAs.

associated differential expression of proteins in tumors compared to adjacent normal tissues. The authors found an age-associated proteomic signature in CRC tumors, which included *MYC*, *E2F*, and *mTORC1* targets, and proteins controlling the *G2M* checkpoint, DNA repair, and unfolded protein response (UPR) pathways expressed at higher levels in older CRC patients. Overall, 208 proteins were found to positively correlate with age, and only 20 negatively correlated with age. Many of these proteins reside in pathways that are targetable with known cancer drugs, supporting the potential use of specific cancer treatments for different ages of CRC patients[87]. For example, the proteins *PIN1*, *ROCK1*, and *ANXA5* are expressed higher in EOCRC and are targetable by Food and Drug Administration (FDA)-approved drugs or drugs in clinical trials[87]. While this study demonstrated the difference in proteomic signatures in younger and older CRC tumors, it was limited by the sample size of approximately 50 total patients with young, intermediate, or older onset CRC[87].

Another recent study by Holowatyj *et al*[74] found no significant differences (FDR q-value < 0.05) in the plasma proteome of younger-onset (n = 11) compared with older-onset (n = 45) CRCs using an antibody microarray platform to detect 206 inflammatory proteins. An increased sample size may shed light on interesting targets, as the authors found that the cancer-related proteins *BRCA2*, *PTEN*, *WNT5B*, and *WNT7A*, among others, had a fold change around two (P < 0.05) in EOCRC *vs* LOCRC serum[74]. While to our knowledge, no other proteome-wide studies have assessed EOCRC, some studies have identified individual proteins that are uniquely expressed in EOCRC tumors. For example, overexpression of the *ALDH1/ALDH1A1* protein has been identified in most EOCRC tumors compared with LOCRCs (Figure 2)[88], and an increase of β-catenin in the nucleus and cytoplasm of EOCRC compared with more membrane staining in LOCRC was shown *via* immunostaining[38].

Protein modifications such as glycosylation[89], ubiquitination[90], phosphorylation, and acetylation [91] are associated with CRC, but age-related characterizations remain limited. A recent study found that an increase in glycosylated hemoglobin in the serum of younger non-diabetic adults correlated with an increased risk for CRC[92], though no studies could be found that focused on post-translational modifications within EOCRC tumors. In addition to changes in protein modifications and expression changes, disruptions to protein folding are common in cancers, eliciting the UPR, which promotes cancer cell survival[93]. Indeed, our previous work showed enrichment of the UPR gene set in EOCRC tumors compared with adjacent control samples[50].

EOCRC in non-western countries

While many studies focus on profiling EOCRC in western countries, the incidence of EOCRC is also increasing in many Asian countries or regions such as Korea, Thailand, Japan, India, and Hong Kong [94,95]. While India reports one of the lowest rates of CRC incidence in the world[94], over half of the sporadic rectal cancers in this country are diagnosed in patients under 50-year-old[96-98]. In addition, studies from Indian cohorts found that under half of early-onset sporadic rectal cancer (EOSRC) tumors exhibit a Wnt signature, the most common driver of CRC, indicating distinct tumor drivers in this population[97,99]. Tumors without Wnt signaling showed increased activation of calcium/nuclear factor of activated T-cell signaling compared to EOSRC with high Wnt signaling[97]. Molecular studies in Indian EOCRC patients have also found a decrease in *KRAS* mutations[99] and deregulation of

MAPK and *PI3K/AKT* pathways[100] compared with LOCRC patients. Whether EOSRC in Indian patients is molecularly distinct from Western or Caucasian patients, from whom most available CRC data were collected, remains unclear.

A recent study by Xu *et al*[43] compared germline mutations in a Western Caucasian EOCRC cohort to a Chinese EOCRC cohort and found the Chinese cohort had significantly fewer hereditary syndromes, with no germline *APC* mutations (mutated in 13% of the western cohort) or *BRCA1*, *SMAD4*, or *CHEK2* mutations, while these genes were mutated in 16% of western cohort patients under 50-year-old (330 Chinese and 430 Caucasian)[43]. Another recent molecular study in China examined clinical information for 947 EOCRC and 3521 LOCRC and found that EOCRCs were more likely to have a family history of cancer, higher TNM stage, and higher 3-year overall survival, but also a lower 3-year disease-free survival[101]. EOCRCs were also more likely to have defective MMR[101], though it is unclear whether this is a product of Lynch syndrome or sporadic mutations.

While data on CRC age-of-onset are available from many European and Asian countries, limited information on CRC epidemiology is available from countries in Africa[94]. New data identified an increased prevalence of CRC, with most African countries where data is available reporting an average age of CRC diagnosis between 43-year-old and 46-year-old[102]. One study compared EOCRC in Nigerians and African Americans (AA) and found that over 60% of Nigerian CRC patients were diagnosed before the age of 50-year-old, compared with 13.2% of AA[103]. The authors identified many differences between the two populations, where Nigerian EOCRCs were younger and had more rectal cancers[103]. Unfortunately, the demographic patterns of EOCRC in black individuals remain severely understudied[103].

In the United States, a clear racial disparity in CRC diagnosis and treatment exists, where the median age of CRC diagnosis is 68 for whites and 64 for blacks[1]. A study by Galadima *et al*[104] found that young AA had higher CRC incidence compared with young individuals of other races[104]. In addition, EOCRC rates in the United States are highest among Indigenous and black Americans[105]. Unfortunately, non-Hispanic blacks with EOCRC have a significantly worse 5-year survival than their white counterparts[106,107]. Previous studies have demonstrated ethnicity-specific differences in underlying CRC mutations[108], but few have focused on EOCRC, especially in people of African descent. One study did find a decrease in the prevalence of *APC* mutations and an increase in gene methylation in an AA EOCRC cohort compared to the mostly white CRC dataset provided by The Cancer Genome Atlas [109].

Overall, the incidence of CRC is increasing in young patients on a global scale, likely due to dietary and lifestyle changes across the world[94]. EOCRC may present differently and have different mutations in different populations around the world, likely owing to both lifestyle and genetic differences[43,97,103,108]. Therefore, it is crucial to increase our understanding of unique EOCRC drivers and to translate this knowledge to improve clinical outcomes for patients worldwide.

EOCRC biomarkers

The majority of EOCRCs are diagnosed between the ages of 40-49[110], leading to the American Cancer Society lowering the recommended CRC screening age from 50 to 45 in 2018[19]. However, several concerns remain on the cost/benefit analysis of this decision[111], indicating a crucial need for cost-effective early screening options.

While colonoscopy remains the gold standard of CRC screenings, blood and fecal tests are cheaper and less invasive options. Blood-based miRNA and DNA methylation biomarkers have been shown to accurately identify EOCRC[112]. There is currently one FDA-approved blood-based CRC screening test, Epi proColon®, which measures methylation of the gene *SEPT9* in cell-free DNA in serum. A recent study showed that methylation of *SEPT9* could accurately distinguish EOCRC patients from healthy controls, indicating that this test is effective for younger patients[112]. Another study suggested that the DNA repetitive elements *LINE-1*, *Sat2*, and *Alu* are hypermethylated in the white blood cells isolated from EOCRC patients, providing an additional potential methylation biomarker signature[62]. In addition to DNA methylation, miRNA expression is gaining popularity as a potential blood-based cancer biomarker. A recent study identified a miRNA signature of four miRNAs that could distinguish both EOCRC and LOCRC serum from healthy controls (Table 3)[75]. Serum expression of inflammatory genes has also been suggested to identify EOCRC patients, and one study found that the chemokine *CXCL12* has lower expression in younger compared to older patients[74].

Another minimally invasive screening option is a fecal test, such as Cologuard™, which measures methylation of the genes *BMP3* and *NDRG4* and assesses samples for the *KRAS* mutation. Cologuard also includes a fecal immunohistochemical test (FIT), which measures human globin, or blood, in the stool (Figure 2). Studies of whether these biomarkers can detect EOCRC are limited, though recent studies have found no significant difference in these markers within CRC tumors in younger and older patients[113-115]. Therefore, while Cologuard and FIT may be effective to detect EOCRC in fecal samples, additional studies are required before such recommendations can be made.

Gene expression biomarkers within tumors have also been suggested to serve as prognostic indicators. *miR-31-5p* was found to be uniquely overexpressed in sporadic EOCRC tumors *vs* normal samples, while it was not overexpressed in LOCRC. Moreover, the *miR-31-5p* target, *DMD*, was also shown to be decreased in EOCRC tumors, and this change in expression correlated with a worse

Table 3 Early-onset colorectal cancer biomarkers

Name	Type	Description
mSEPT9	Methylation, DNA	Blood-based biomarker used in Epi proColon® or both EOCRC and LOCRC[112]
miR-193a-5p, miR-210, miR-513a-5p, miR-628-3p	miRNAs	miRNA in serum, panel works for both EOCRC and LOCRC[75]
Sat2, LINE-1, Alu	Methylation, DNA	DNA repetitive elements with increased methylation in EOCRC in white blood cells[62]
miR-31-5p, DMD	miRNA, mRNA	Transcripts uniquely overexpressed in sporadic EOCRC tumor *vs* normal and not in LOCRC. *miR-3105p* targets *DMD* and it's downregulated in EOCRC[116]
MYC	mRNA	Transcription factor with increased tumor expression in EOCRCs may subset patients into distinct groups[50,69]

EOCRC: Early-onset colorectal cancer; LOCRC: Later-onset colorectal cancer; miRNA: microRNAs.

prognosis (Table 3)[116]. In addition to genetic, transcriptomic, and proteomic alterations, biomarkers of the gut microbiome have also been suggested to identify EOCRC, as EOCRC has been shown to have a distinct microbiome compared with LOCRC[117]. Microbiota studies in CRC and EOCRC are outside the scope of this review but are nicely summarized by Abdullah *et al*[118]. Overall, a limited number of studies have shown that common CRC screening options may apply to EOCRC as well. miRNA and DNA methylation biomarkers have been proposed to help identify EOCRC (Table 3), however additional studies with larger sample sizes and clinical validations are required.

Currently, clinical practice guidelines do not differentiate the treatment of EOCRC *vs* LOCRC[119]. However, CRC treatment is dependent on tumor mutational profiling, and thus, the lower frequency of *BRAF*[34,38,57] and *KRAS*[99,120] mutations in EOCRC means the mutation-specific treatments will be less commonly used in EOCRC patients. The efficacy of these drugs in EOCRC has not been directly studied but the mechanism is likely very similar to LOCRC.

CONCLUSION

Several clinical features have been associated with EOCRC. Approximately 75% of sporadic cases occur in the 40–year-old to 49-year-old age group, with 55%-80% of EOCRCs occurring in the distal colon or rectum[121,122]. The increasing rate of EOCRC has been predominated by an increasing rate of distal colon and rectal cancer, with individuals born circa 1990 having double and quadruple the risk of colon and rectal cancer, respectively, compared to those born circa 1950[123]. While many strides have been made to understand alterations at the DNA, RNA, and protein levels that contribute to EOCRC, questions remain on how EOCRC patients should be treated compared with their older counterparts. Currently, young patients are more likely to be treated, or overtreated, with systemic chemotherapy, but have similar clinical outcomes compared with older patients[10,104,124].

As the number of EOCRC cases continues to rise globally, there is a critical need to optimize cancer treatment strategies, as well as to further develop non-invasive screening options to identify people at risk for EOCRC. Currently, scientific studies are limited by low sampling size, especially in non-white patients. Researchers are addressing this limitation by continuing to grow biobanks with younger and non-diseased samples. Furthermore, machine learning approaches have been suggested to increase the statistical power of limited sample sizes[125], which could be applied to identify EOCRC risk genes or genetic loci in under-represented minorities. Another limitation is the lack of a clear model system to test hypotheses on EOCRC. While models for EOCRC exist, as *APC*^{min} mice develop CRC at a young age, and HCT-116 cells are from a young patient[126], these systems fail to recapitulate the diversity in EOCRC subtypes that are observed in patient samples. The development of stable cell lines from CRC samples generally requires transformation, altering the cellular profiles, and limiting normal controls. Organoid models are gaining popularity due to their ability to recapitulate the colonic crypt structure from both normal and tumor cells[33,127]. Future work will tease out the molecular mechanisms unique to EOCRC with growing biobanks and organoids as well as other innovative model systems.

FOOTNOTES

Author contributions: Marx O and Mankarious M collected the data; Marx O, Mankarious M, and Yochum G wrote the paper.

Conflict-of-interest statement: All the authors report having no relevant conflicts of interest for this article.

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S-Editor: Li L
L-Editor: Filipodia
P-Editor: Li L

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World Journal of Biological Chemistry

Submit a Manuscript: https://www.f6publishing.com

World J Biol Chem 2023 March 27; 14(2): 28-39

DOI: 10.4331/wjbc.v14.i2.28

ISSN 1949-8454 (online)

MINIREVIEWS

Anticancer potential of *Ferula assa-foetida* and its constituents, a powerful plant for cancer therapy

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Sekhar P, India; Thongon N, Thailand

Received: November 10, 2022
Peer-review started: November 10, 2022
First decision: January 20, 2023
Revised: January 24, 2023
Accepted: February 21, 2023
Article in press: February 21, 2023
Published online: March 27, 2023

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Abstract

Cancer is one of the main challenges of the health system around the world. This disease is increasing in developing countries and imposes heavy costs on patients and governments. On the other hand, despite various drugs, the death rate among cancer patients is still high and the current treatments have many harmful effects. In the traditional medicine of different countries, there are many medicinal plants that can be effective in the treatment of cancer. Ferula plants are traditionally used as spices and food or for medicinal purposes. *Ferula assa-foetida* is one of the famous plants of this genus, which has been used for the treatment of various diseases since ancient times. Among the main compounds of this plant, we can mention monoterpenes, sulfide compounds and polyphenols, which can show different therapeutic effects. This article has been compiled with the aim of collecting evidence and articles related to the anti-cancer effects of extracts, derived compounds, essential oils and nanoparticles containing *Ferula assa-foetida*. This review article was prepared by searching the terms *Ferula assa-foetida* and cancer, and relevant information was collected through searching electronic databases such as ISI Web of Knowledge, PubMed, and Google Scholar. Fortunately, the results of this review showed that relatively comprehensive studies have been conducted in this field and shown that *Ferula assa-foetida* can be very promising in the treatment of cancer.

Key Words: *Ferula assa-foetida*; Anticancer; Essential oil; Isolated components; Nano particle; Extract

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Core Tip: Finding new anti-cancer compounds is an important necessity in the treatment or prevention of this disease. *Ferula assa-foetida* has useful compounds for the prevention and treatment of cancer, which can be used in making new compounds. These compounds include sulphide compounds, flavonoids and terpene coumarins, which with new methods such as making emulsions and nanoparticles from these compounds can be of great help in reducing the costs of cancer patients and their life expectancy.

Citation: Sirizi MAG, Alizadeh Ghalenoei J, Allahtavakoli M, Forouzanfar H, Bagheri SM. Anticancer potential of *Ferula assa-foetida* and its constituents, a powerful plant for cancer therapy. *World J Biol Chem* 2023; 14(2): 28-39

URL: https://www.wjgnet.com/1949-8454/full/v14/i2/28.htm

DOI: https://dx.doi.org/10.4331/wjbc.v14.i2.28

INTRODUCTION

Today, one of the main problems of the health community is cancer, which is currently known as the second leading cause of death in the world. The most common cancers are breast and lung cancer worldwide, accounting for 12.5% and 12.2% of all newly diagnosed cases, respectively[1]. Common treatments include radiotherapy and chemotherapy that stop the cell cycle through apoptosis or non-apoptosis mechanisms such as necrosis[2]. These therapies have a variety of side effects, including damage to healthy cells. Medicinal plants have therapeutic value due to their biologically active compounds such as terpenes, coumarins, phenolic and alkaloids[3]. These natural compounds have shown promising insight into the treatment and prevention of cancer by restricting the division of tumor cells or inducing apoptosis with the advantage to reduce side effects[4]. The genus Ferula includes 170 different species that are distributed all over the world and this genus belongs to the Apiaceae (Umbelliferae) family[5]. *Ferula assa-foetida*, one of the famous species of Ferula that is used in Iranian traditional medicine for the treatment of digestive diseases, nervous problems and some reproductive system disorders such as decreased libido[6]. Asafoetida or Anghouzeh (Traditional name in Persian), is an oleo gum resin which obtained from the root of *Ferula assa-foetida* and traditionally used as anthelmintic, anticonvulsant, sexual aphrodisiac and analgesic agent[7]. New scientific reports have shown that asafoetida has antifungal[8], antidiabetic[9], antiinflammatory[10], antimutagenic[11] antidementia[12], anticonvulsant[13], antiviral[14], anti-cancer[15] and relaxant[16] activities and also has preventive effect against cuprizone induced demyelination[17]. There is not enough information available about the dosage and toxicity of asafoetida, but it is recommended not to consume more than 0.2 g per day[18], and it has also been shown that long-term and high-dose administration (200 mg and above) causes liver damage[19]. The main compounds that have been identified in the *Ferula assa-foetida* include glycoside compounds, various terpenoid, coumarin derivatives, and sulfide compounds[20,21] which have been shown to have anti-cancer potential (Figures 1 and 2).

Some compounds isolated from *Ferula assa-foetida* have also been shown to have various pharmacological properties. For example, Ferulic acid is one of these compounds that has antioxidant and neuroprotective properties[22]. Umbelliferon is a coumarin compound that has antioxidant and antidiabetic as well as antitumor effects[23]. In recent years, many studies have been conducted on the anti-cancer effects of Ferula. The members of this genus have shown high anti-cancer potential, which can provide a good basis for finding new anti-cancer agents. Our focus on published studies on the impact of different extracts and compounds isolated from *Ferula assa-foetida* as anticancer agents. Due to the increase in cancer patients and significant findings on the anticancer effects of *Ferula assa-foetida*, this article is designed for help to researchers finding new anticancer compounds.

METHOD

This review article was prepared by searching the terms of *Ferula assa-foetida* and cancer. Information about *Ferula assa-foetida* and its anticancer effect was collected on electronic databases including ISI Web of Knowledge, Medline/PubMed, ScienceDirect, Embase, Scopus, Biological Abstract, Chemical Abstract and Google Scholar. To make the research easier to understand, the article is divided into different sections, including the anti-cancer effects of nanoparticles containing *Ferula assa-foetida*, essential oils, extracts, isolated compounds from *Ferula assa-foetida*, and preclinical and experimental studies (Table 1).

Table 1 An overview of anticancer effect of different parts of *Ferula assa-foetida*

	Type/name	Cell line	Effects	Ref.
Nano particle	Silver nanoparticles and asafoetida ethanol extracts	L6 cancer cell line	IC50 was calculated 1 μg/mL	Subramaniam *et al* [25], 2021
	Nano emulsion containing *Ferula assa-foetida* seed essential oil	MCF7 and A2058 cell line	Increased BAX and decreased BCL2 expression. IC50 = 64 μg/mL for MCF7 and 201 μg/mL for A2058. Also, decreased VEGF at 32 μg/mL and VEGFR at 128 μg/mL	Azani *et al*[26], 2021
	Lipid nanoparticles containing *Ferula assa foetida* seed oil	NT-2 human cancer stem cells	IC50 = 115.4 μg/mL and the number of blood vessels reduced at 250, 500, and 1000 μg/mL	Sadat Khadem *et al* [27], 2021
	Silver anoparticles (AgNPs) with aqueous extract of asafoetida	MCF-7	IC50 was calculated 2 μg/mL	Devanesan *et al*[28], 2020
	Zinc nanoparticles containing *Ferula assa-foetida* extract	MCF7, MDA-MB231 and HT-29	IC50 was 23, 41.26 and 143 μg/mL after 72 h	Boskabadi *et al*[29], 2020
	Ferula assa foetida essential oil on PLGA nanoparticles	HepG2 and A2780	Inhibited HepG2 and A2780 with an IC50 of 57 μg/mL and 106.7 respectively. Reduction of vascular parametric factors at 125 μg/mL	Mokhtareeizadeh *et al*[30], 2021
Essential oil	(-)-E-2-butylpropenyl disulfide, (-)-Z-2-butylpropenyl disulfide, (-)-1-(methylthio) propyl (E)-1 -Propenyl disulfide, and (-)-1-(methylthio) propyl (Z)-1-propenyl disulfide	SKOV3 (ovary) and A549 (lung) cancer cell lines	Trisulfide showed better activity against A549 and SKOV3 cell lines compared to disulfides	Yatham *et al*[31], 2021
	Seed of *Ferula assa foetida* essential oil	AGS gastric cancer cells	Inhibitory effect on AGS gastric cancer cells was near 100% at 10 μl/mL after 72 h incubation	Bagheri *et al*[32], 2020
	Asafoetida essential oil	HepG2 and SK-Hep1	IC50 for HepG2 and SK-Hep1 was 7.21 μg/mL and 8.0 μg/mL respectively	Verma *et al*[33], 2019
	Essential oils asafoetida and	T98G and HCT116	IC50 value for HCT116 was 5.96 μg/mL and for T98G was 4.49 μg/mL	Pavela *et al*[34], 2020
	Essential oil of asafoetida	MCF7 cells	Decreased the viability of MCF7 cells in a time and concentration-dependent manner	Bagheri *et al*[35], 2020
Isolated components	Ferulic acid	MDA-MB-231	Combination with 25 μM of thymoquinone and 250 μM of ferulic acid, decrease proliferation of MDA-MB-231 cells	Al-Mutairi *et al*[38], 2021
	Ferulic acid	MDA-MB-231	Increased caspase 3 and reduced the proliferation of cancer cells about 40% at 100 μM. 100 mg/kg significantly reduced tumor volume, weight and growth in mice	Zhang *et al*[39], 2016
	Ferulic acid	4T1 cells	Reduced the growth of cancer cells at 500 μg/mL	Bagheri *et al*[40], 2017
	Galbanic acid	MDA-MB-231 and MCF-7 cells	IC50 was 48.7 and 56.6 μg/mL, respectively. Up-regulation of Bax and caspase-3 and down-regulation of bcl2	Sajjadi *et al*[42], 2019
	Galbanic acid	H460, A549, PC-9 and HCC827	IC50 calculated 100 μM on H460 cell line. Bax and caspase 9 increased and Bcl-2, Bcl-xL and myeloid cell leukemia 1 (Mcl-1) decreased in H460 cells	Oh *et al*[43], 2015
	Galbanic acid	AR+ PCa cells and AR- PCa cells	Suppresses the growth of AR (+) PCa cells. Inhibited cyclin/CDK4/6 pathway, specially cyclin D1	Zhang *et al*[44], 2012
	Farnesiferol C	HUVEC and mouse Lewis lung cancer cells	10-40 μmol/L inhibited VEGF. Reduced the growth of mouse Lewis lung cancer by 60%	Lee *et al*[45], 2010
	Sesquiterpene coumarins	PC-3 and MCF-7	Gummosin showed highest cytotoxic activity. Also showed an IC50 values at 30 and 32.1 μg/mL against PC-3 and MCF-7 cell lines respectively	Iranshahy *et al*[48], 2019
	Farnesiferol C	MCF-7	Decrease cell viability after 24, 48 and 72 h. (IC50 43, 20 and 14 μM, respectively), and stopped the cell cycle in G0/G1 phase and induced apoptosis in MCF-7 cells	Hasanzadeh *et al* [46], 2017

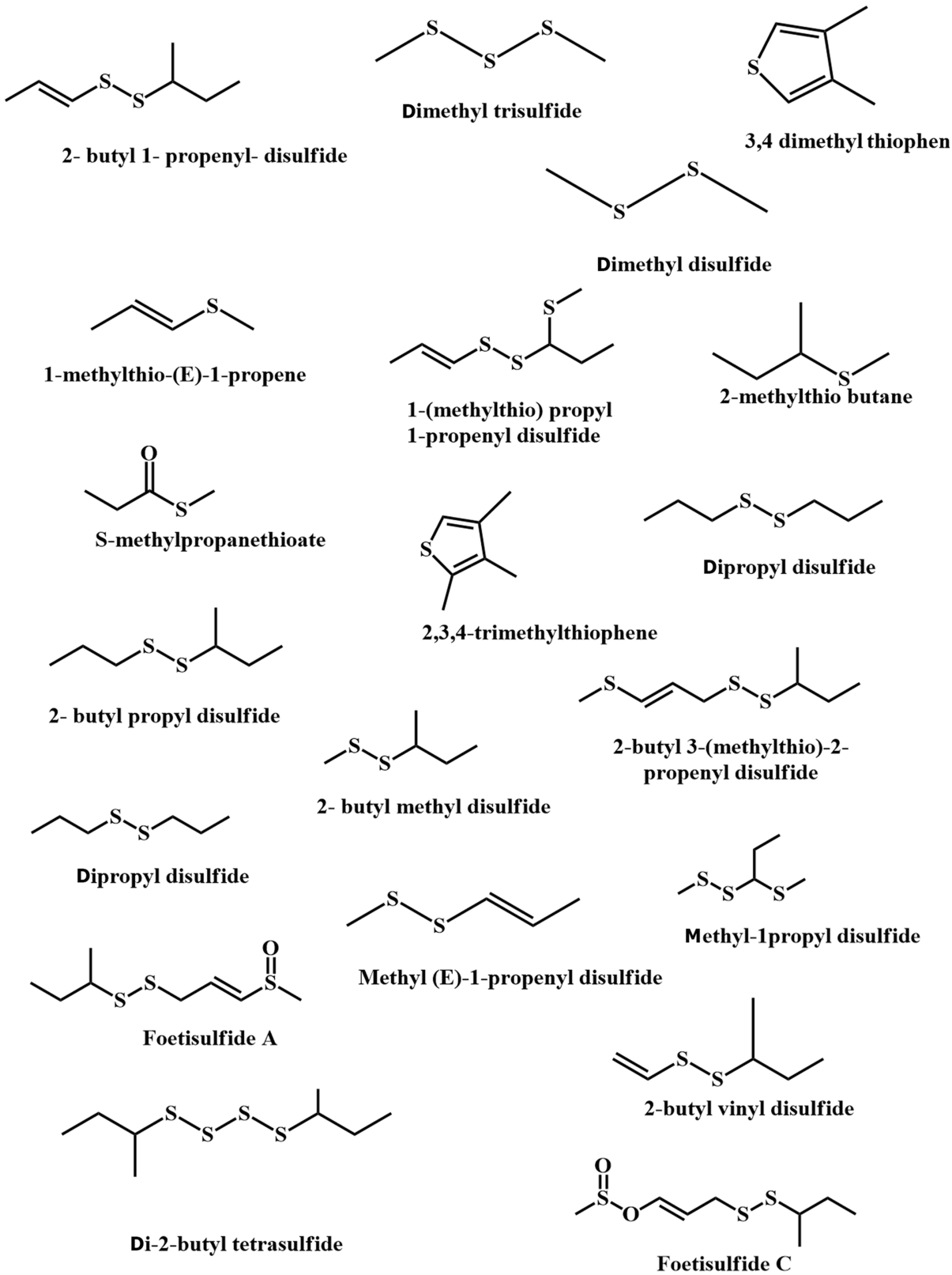
AGS: Aerobic granular sludge; BCL2: B-cell lymphoma 2; EMT: Epithelial-mesenchymal transition; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor.

ANTICANCER EFFECT OF NANOPARTICLES CONTAINING *FERULA ASSA-FOETIDA*

Encapsulation of essential oils, extracts and plant derivatives can overcome their therapeutic limitations and lead to better stability, increased bioavailability and better efficacy[24]. The use of nanoparticles in cancer treatment is a new method that can be used to target treatment. *Ferula assa-foetida* has various biological compounds that make it a suitable candidate for use in cancer treatment. Various studies have been conducted on the effect of different derivatives and extracts of this plant on different cell lines of cancer cells and generally positive results have been obtained. For example, use of silver nanoparticles and ethanol extract of asafoetida caused a decrease in the survival rate of L6 cancer cells, and the IC_{50} value was calculated as 1 μg/mL[25]. Some studies have shown that nanoemulsion containing *Ferula assa-foetida* essential oil can cause apoptosis by increasing BAX expression and decreasing BCL-2 in MCF7 cancer cells. The lethality of this nanoparticle has been calculated based on IC_{50} equal to 64 μg/mL for MCF7 and 201 μg/mL for A2058. Also, a significant decrease in the expression of vascular endothelial growth factor (VEGF) at 32 μg/mL and vascular endothelial growth factor receptor (VEGFR) at 128 μg/mL was observed in MCF-7 cells treated with nanoemulsion. This nanoparticle was able to significantly reduce tumor indices in the murine model of induced breast cancer at a concentration of 100 mg/kg[26]. Lipid nanoparticles containing *Ferula assa-foetida* seed oil on NT-2 human cancer stem cells had an IC_{50} equal to 115.4 μg/mL. The morphometric results of blood vessels treated with these nanoparticles showed that the number of blood vessels was significantly reduced in concentrations of 250, 500 and 1000 μg/mL in a dose-dependent manner. Also, these nanoparticles increased the expression of TNF-α, P21, and Cas3[27]. Synthesis of silver nanoparticles (AgNPs) with aqueous extract of asafoetida on MCF-7 cells caused cell death in a dose-dependent manner and its IC_{50} was calculated as 2 μg/mL[28]. By making zinc nanoparticles containing *Ferula assa-foetida* extract and investigating its effects on MCF7, MDA-MB231 and HT-29 cell lines, Boskabadi *et al*[29] showed that this nanoparticle can significantly reduce the growth of cancer cells. The calculated IC_{50} was equal to 23, 41.26 and 143 μg/mL after 72 h, respectively. In addition, the results showed that the nanoparticle has apoptotic properties and antioxidant activity with an IC_{50} equal to 500 mg/mL. Expression of Bax and Bcl2 significantly up and down regulated respectively. Mokhtareeizadeh *et al*[30] founded that nanoparticles containing *Ferula assa-foetida* essential oil can inhibit the growth of HepG2 and A2780 cells with IC_{50} of 57 and 106.7 μg/mL respectively. These nanoparticles caused a significant decrease in angiogenesis in fertilized eggs at a dose of 125 μg/mL. Also it induced apoptosis and death of cancer tissue cells by regulating Caspase3 and 9, TNF-α, P53 and P21 in nude mice with breast cancer.

ANTICANCER EFFECT OF ESSENTIAL OIL OF *FERULA ASSA-FOETIDA*

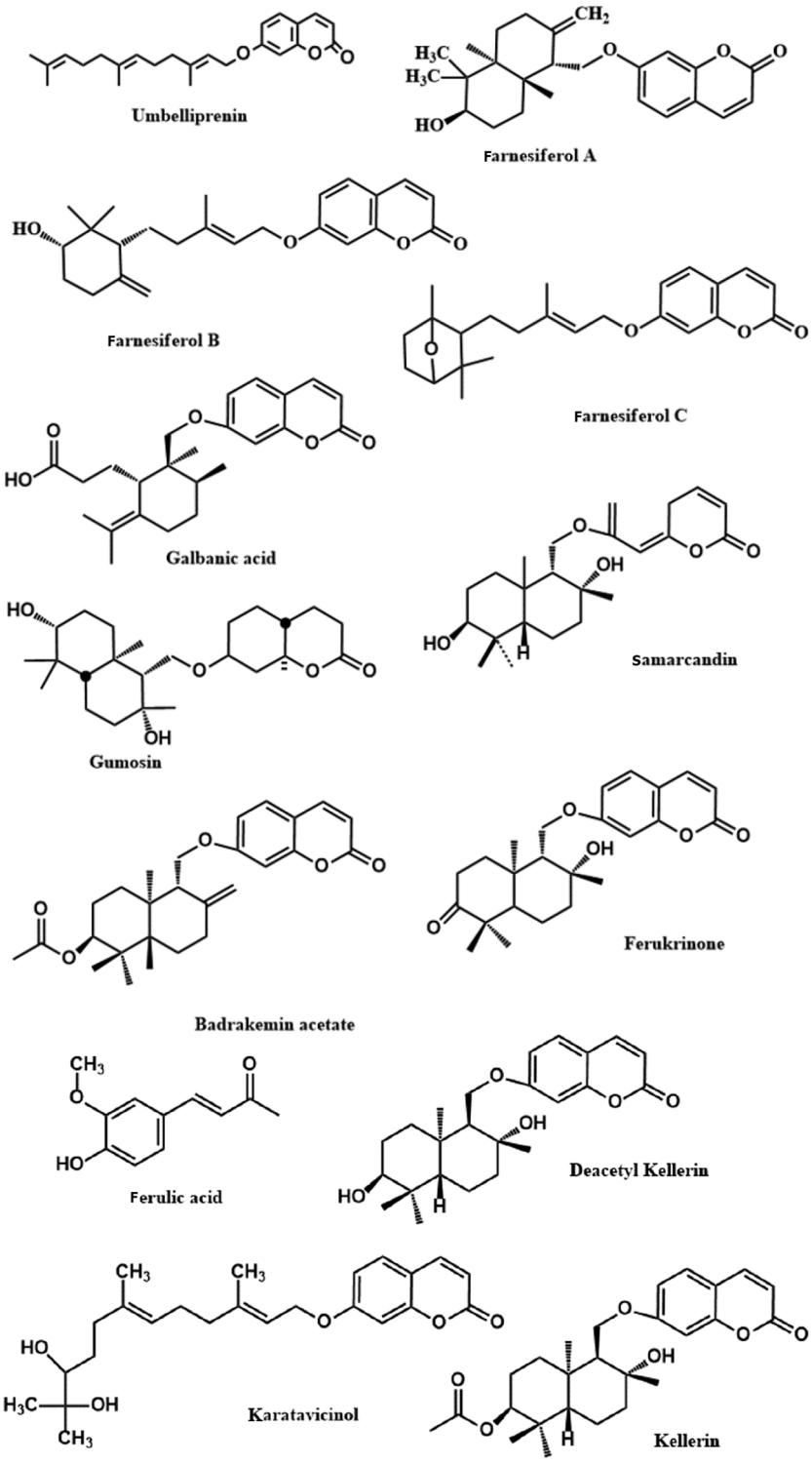
The main part used by *Ferula assa-foetida* is an oleo gum resin, which is obtained by shaving its root. This oleo gum resin contains many different compounds, the anti-cancer effects of some of these compounds have been investigated. The volatile part of oleo gum resin or its essential oil contains generally sulfur compounds that have a pungent and unpleasant smell. Some studies have shown that essential oil has strong anti-cancer effects. For example, Yatham *et al*[31] found four main compounds in asafoetida essential oil, including (-)-E-2-butylpropenyl disulfide, (-)-Z-2-butylpropenyl disulfide, (-)-1-(methylthio) propyl (E)-1 -Propenyl disulfide, and (-)-1-(methylthio) propyl (Z)-1-propenyl disulfide were identified and investigated their potential to inhibit the growth of cancer cell lines SKOV3 (ovary) and A549 (lung). Meanwhile, trisulfide showed better activity against A549 and SKOV3 cell lines compared to disulfides. The analysis of *Ferula assa-foetida* seed essential oil showed that it contains compounds such as E-1-propenyl sec-butyl disulfide (13.13%) Z-1-propenyl sec-butyl disulfide (11.34%). This essential oil exerted its inhibitory effect on aerobic granular sludge gastric cancer cells near 100% in 10μl/mL in 72 h after incubation[32]. The anti-proliferative and anti-apoptotic effects of asafoetida essential oil on liver cancer cell lines (HepG2 and SK-Hep1) as well as the expression of NFKB1, TGFB1, TNF, and caspase3 genes showed that the IC_{50} of the oil for HepG2 and SK-Hep1 was 7.21 μg/mL and 8.0 μg/mL respectively. After EO treatment, the genes involved in metastasis and proliferation decreased and the genes involved in apoptosis showed a significant increase (casp3 and TNF). Analysis of the essential oil by GC showed the presence of 1, 2-dithiolane in the amount of 87.4%[33]. Pavela *et al*[34] evaluated the essential oils asafoetida and *Ferula gummosa* on T98G (human glioblastoma multiforme cell line), HCT116 (human colon cancer cell line). *Ferula assa-foetida* essential oil was more active on HCT116 with IC_{50} value of 5.96 μg/mL and *Ferula gummosa* essential oil showed more activity on T98G with IC_{50} value of 4.49 μg/mL. Essential oil of asafoetida (EOA) exposed MCF7 cells to different concentrations of EOA (2, 4, 6, 8, and 10 μl/mL) at 24, 48 and 72 h showed that EOA significantly decreased the viability of MCF7 cells in a time and concentration-dependent manner. The



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Figure 1 Chemical structure of some sulfide compounds derived from Ferula assa-foetida.

major constituents identified in EOA were E1propenyl secbutyl disulfide (36.15) and Z1propeny secbutyl disulfide (27.93%)[35]



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Figure 2 Chemical structure of isolated constituents from *Ferula assa-foetida* showed anticancer effect.

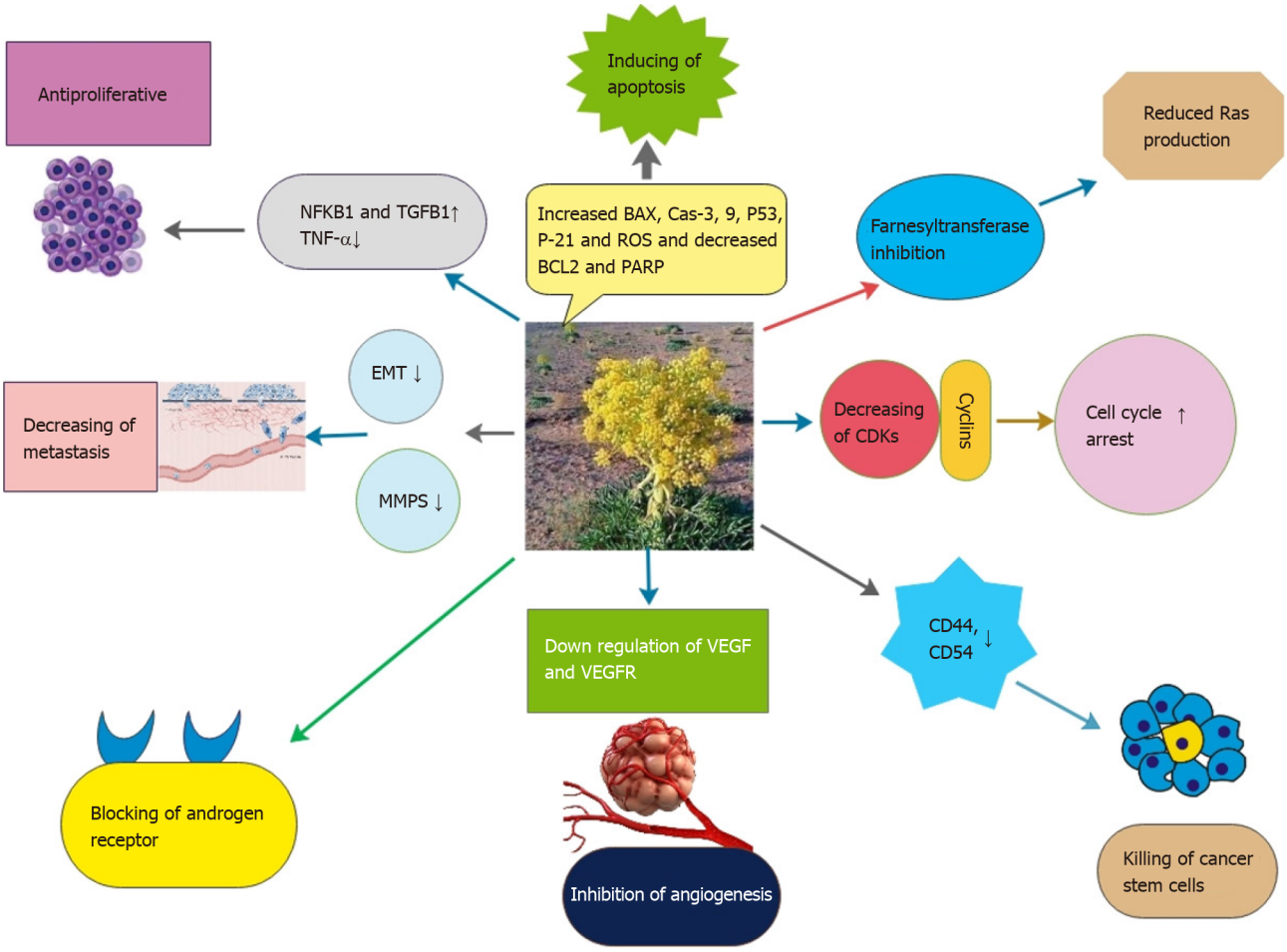
ANTICANCER EFFECT OF ISOLATED CONSTITUENTS FROM *FERULA ASSA-FOETIDA*

Several compounds are derived from *Ferula assa-foetida*, which include coumarins, sesquiterpene coumarins, flavonoids and phenolic constituents that have shown a number of pharmacological effects, including antibacterial, antifungal, cytotoxic, antioxidant and hormonal activities, as well as anticancer effects[36]. Ferulic acid is one of the phenolic compounds in asafoetida, which has various therapeutic effects[37]. Al-Mutairi *et al*[38] have shown that when ineffective doses of ferulic acid were used with ineffective doses of thymoquinone, it was able to significantly reduce the death of MDA-MB- cells after 48 h. In another study, ferulic acid increased caspase 3 activity in the breast cancer cell line MDA-MB-231 and reduced the proliferation of the cancer cell line about 40% after 72 h at a concentration of 100

μM. Also, the anti-tumor potential of ferulic acid in a xenograft mouse model with MDA-MB-231 at a concentration of 100 mg/kg body weight could reduce tumor volume, weight and growth[39]. Bagheri *et al*[40], showed that ferulic acid significantly reduced the growth of 4T1 mouse breast cancer cells at a dose of 500 μg/mL. Galbanic acid is a terpenes lactone derived from the gum of *Ferula assa-foetida*, which has also been identified in several other species of Ferula[41]. Treatment of MDA-MB-231 and MCF-7 cells with galbanic acid showed that this compound leads to the inhibition of proliferation and induction of apoptosis with IC_{50} of 48.7 and 56.6 μg/mL, respectively. Also, galbanic acid stimulated apoptosis through the up-regulation of Bax and caspase-3 and the down-regulation of bcl2 and increased the expression of superoxide dismutase, catalase and glutathione peroxidase genes[42]. In confirmation of these results, in another study, the potential of galbanic acid in inhibiting four types of non- small lung cancer cells H460 and A549, PC-9 and HCC827 were proven after 24 h. Meanwhile, H460 cell line has the highest sensitivity to galbanic acid and showed an IC_{50} of about 100 μM. It was also found that the expression levels of Bax and caspase 9 increased and Bcl-2, Bcl-xL and myeloid cell leukemia 1 (Mcl-1) decreased and cleaved poly (ADP-ribose) polymerase (PARP) in H460 cells[43]. Androgen receptor (AR) signaling is crucial for the initiation and progression of prostate cancer (PCa). In a study, it was found that galbanic acid preferentially suppresses the growth of AR (+) PCa cells compared to AR (-) PCa cells. Galbanic acid induces apoptosis through G1 arrest associated with inhibition of cyclin/CDK4/6 pathway, especially cyclin D1[44]. The anti-angiogenic activities of farnesiferol C (FC) in human umbilical vein endothelial cells showed that exposure to a concentration range of 10-40 μmol/L FC inhibited VEGF, migration, invasion cells and decrease the expression of matrix metalloproteinase 2. Furthermore, FC inhibited the angiogenesis of mouse aorta treated with VEGF in an experimental model. FC reduced the growth of mouse Lewis lung cancer by 60% and caused rapid inhibition of VEGFR1 autophosphorylation caused by VEGF without affecting VEGFR2. However, FC inhibited the phosphorylation of most VEGFR2 downstream kinases such as focal adhesion kinase, Src, extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinase, and c-jun-NH2-kinase without affecting AKT[45]. Sesquiterpene coumarins are a group of compounds found in the genus Ferula that have shown various therapeutic effects such as anticancer effects[21]. Farnesiferol C obtained from the chloroform extract of *Ferula assa-foetida*, on MCF-7 cells, led to a decrease in cell viability after 24, 48 and 72h. (IC50 43, 20 and 14 μM, respectively). Farnesiferol C stopped the cell cycle in G0/G1 phase and induced apoptosis in MCF-7 cells. This compound increased cellular SOD, CAT MDA activities in 24 and 48 h and reduced activity of SOD and CAT and increased MDA level after 72 h exposure. It demonstrated that reactive oxygen species level increased 5.92%, 13.53% and 14.43% after 24, 48 and 72 h exposure, respectively[46]. Treatment of K562, KBM5, U937 and HL-60 cancer cells with farnesiferol C showed that this substance has an IC_{50} = 10 μM on K562 cells and 20μM on KBM5 cells and showed a significant effect only on these two types of cells. Also, cleaved PARP and caspase 3 and 9 decreased the expression of Bcl2 and stopped cells in G1, and farnesiferol C decreased the expression of Cyclin D1, Cyclin E, Cyclin B1 and histone deacetylase 1 and 2 in K562 and KBM52 cells[47]. Investigation on anticancer potential of ten sesquiterpene coumarins include farnesiferol A, farnesiferol B, farnesiferol C, gummosin, samarkandin, umbelliprenin, badrakemine acetate, ferukrinone, kellerin and deacetyl kellerin derived from asafoetida showed that gummosin has highest cytotoxic activity among these sesquiterpene coumarins. It showed an IC_{50} values of 30 and 32.1 μg/mL against PC-3 and MCF-7 cell lines respectively[48]. Umbelliprenin is a prenylated coumarin compound found in Ferula species, also isolated from *Ferula assa-foetida*. This structure has various pharmacological effects such as cytotoxic activities and induction of apoptosis[49]. Using the umbelliprenin isolated from *Ferula assa-foetida* on Jurkat T-CLL and Raji B-CLL cell lines showed that umbelliprenin induced apoptosis in a dose- and time-dependent manner (IC50, 16 h = 75 μM and 48 h = 25 μM respectively)[50]. Farnesylation of the activated oncogenic ras product by Farnesyltransferase (FTase) is a critical step for its oncogenic function. Isolation of galbanic acid, karatavicinol, umbelliprenin, farnesiferol B, farnesiferol C from *Ferula assa-foetida* to inhibit FTase showed that galbanic acid has the highest enzyme inhibition potential and IC_{50} was calculated as 2.5 μM. In addition, the calculated IC_{50} value in reducing the proliferation of oncogenic ras-transformed NIH3T3/Hras-F cells by galbanic acid was 16.2 μM compared to the control group[51].

DIFFERENT EXTRACTIONS OF *FERULA ASSA-FOETIDA* ON CANCER

Ferula assa-foetida ethanolic extract showed a significant effect on PC12 and MCF7 cells in reducing cell survival. The amount of IC_{50}s for 24, 48 and 72 h for MCF7 was 1.30, 1.284, 0.753 μM, respectively. Also, IC_{50}s for PC12 category at 24, 48 and 72 h were calculated as 2.84, 0.8 and 0.4 μM, respectively[52]. The petroleum benzene, chloroform and methanol extract of asafoetida on MCF7 HepG2, A549, HT-29 and MDBK showed that the methanol fraction has an IC_{50} of more than 100 μg/mL. Petroleum and chloroform extracts showed IC_{50} values less than 52 μg/mL in four cell lines. Chloroform fraction showed IC_{50} equal to 61.42 μg/mL in MCF7. The petroleum afraction showed an IC_{50} of 45.73 μg/mL in MCF7[53]. The hydroalcoholic extract of *Ferula assa-foetida* significantly reduce the mRNA expression level of epithelial-mesenchymal transition markers (vimentin, Snail1, Zeb1) and the anti-apoptotic



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Figure 3 Investigated mechanisms by which *Ferula assa-foetida* exerts its anticancer effects. BCL2: B-cell lymphoma 2; CDKs: Cyclin-dependent kinases; EMT: Epithelial-mesenchymal transition; MMPS: Matrix metalloproteinases; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor; ROS: Reactive oxygen species.

marker Bcl-2, as well as the expression of stem cell marker CD44 and CD54[54]. Ethanol extracts of *Ferula assa-foetida* and a number of its components (ferulic acid, vanillic acid, quercetin, ellagic acid, and p-coumaric acid) had cytotoxic effects on MCF-7 or MDA-MB-231 human breast cancer cells and 4T1 mouse cell line. Also, THP-1 peripheral blood monocytic leukemia cells can be polarized to M1 inflammatory phenotype by treatment with the extract and its components. Furthermore, this THP-1-dependent polarization of macrophages demonstrated an enhanced ability to damage MCF-7 or MDA-MB-231 cell monolayers in co-culture experiments. Therefore, treatment with *Ferula assa-foetida* extract can also indirectly cause the death of cancer cells through the activation of immune cells[55]. The cytotoxic effects of the ethanolic extract of Ferula assa-foetida resin on HepG2 cell line in concentrations (10, 50, 100, 200 μg/mL) showed that this extract in doses of 50, 100 and 200 μg/mL decreased the viability of HepG2 cells but in doses of 100 and 200, it also changes the shape of normal L929 cells. Therefore, only a dose of 50 μg/mL can be considered as an effective and non-toxic dose[56]. The investigation of methanolic and ethanolic extract of *Ferula assa-foetida* resin on osteosarcoma cell line showed that different concentrations of the extract in 24 and 48 h can reduce the survival of cancer cells. The highest effect rate corresponding to the concentration of 20 mg in 48 h for ethanolic and methanolic extract was calculated as 29.5 and 35.2, respectively. Also, the results showed that the ethanolic extract has a greater effect on the death of cancer cells[57].

ANIMAL EVIDENCES FROM ANTI-TUMOR EFFECT OF *FERULA ASSA FOETIDA*

Although animal evidence for the anticancer effect of *Ferula assa-foetida* is not much, several limited studies have shown that this plant has good anticancer potential. In a study, it was found that the use of 100 mg/kg asafoetida for 21 d against breast cancer caused by 4T1 cells in BALB/c mice can reduce tumor weight and tumor volume and increase the weight of treated mice. Also, asafoetida reduced lung, liver and kidney metastasis respectively. Asafoetida showed significant inhibitory activity against

lipoxygenase as well as antioxidant activity[15]. The use of food containing asafoetida (1.25 and 2.5%) showed that asafoetida significantly restored the level of the antioxidant system MNU (N-methyl-N-nitrosourea) induced mammary carcinogenesis in Sprague-Dawley rats. Furthermore, only in the MNU-control group, all animals had tumors with an average of 5.45 tumors per mouse (tumor burden) at the end of 18 wk, but the tumor burden in treated groups (1.25% and 2.5%) with asafoetida decreased to 3.6 and 2.3 tumor/mouse, respectively. The tumor volume in treated groups also decreased to 1.9cc (40%) and 1.3cc (59%), respectively, compared to 3.2cc in control group[58]. The use of different doses of asafoetida (5, 10 and 20 mg/100 g body weight) on dimethylhydrazine-induced colon cancer in rats showed that body weight, tumor frequency, tumor incidence, tumor size, total serum sialic acid as well as the tissue structure of the colon improved in all groups treated with asafoetida and these effects was better at dose of 10 mg/ 100 g body weight than other doses[59].

ANTICANCER MECHANISMS

The results of this study show that extracts and compounds isolated from Ferula asafoetida can cause the death of cancer cells in different ways. These mechanisms are briefly shown in Figure 3. As can be seen from this diagram, by reducing angiogenesis, increasing apoptosis, inhibiting metastasis, affecting the oxidative system of cancer cells and disrupting the cycle of cancer cells, *Ferula assa-foetida* causes damage and death of these cells.

CONCLUSION

Cancer is one of the serious problems of human society, especially in developing countries. The costs of treating the disease are very high and the death rate caused by it is worrying. The healthcare system and the research community should find effective and low-cost treatment methods as soon as possible, especially for poor communities. Finding anti-cancer compounds of natural origin is one of these solutions. It is very encouraging to see the results of the anti-cancer effects of *Ferula assa-foetida*. These results show that asafoetida can be considered as a medicinal plant in cancer treatment. Many of the effective compounds found in plant gum have anti-cancer effects, which can be inspired by these compounds to create new drugs. The use of asafoetida as a seasoning in foods can also be effective in the follow-up of cancer. By taking advantage of new methods such as nanotechnology and biotechnology, we can imagine a better perspective in using this plant and its derivatives as an anti-cancer agent.

FOOTNOTES

Author contributions: Bagheri SM and Allahtavakoli M designed the research study; Sirizi MAG and Alizadeh Ghalenoei J analyzed the data and wrote the manuscript; Forouzanfar H contributed new reagents and analytic tools; Bagheri SM Final review and editing; All authors have read and approve the final manuscript.

Conflict-of-interest statement: All the author declare no conflict of interests for this article.

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S-Editor: Liu JH
L-Editor: A
P-Editor: Liu JH

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World Journal of Biological Chemistry

Submit a Manuscript: https://www.f6publishing.com

World J Biol Chem 2023 March 27; 14(2): 40-51

DOI: 10.4331/wjbc.v14.i2.40

ISSN 1949-8454 (online)

ORIGINAL ARTICLE

Observational Study

Temporal pattern of humoral immune response in mild cases of COVID-19

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Specialty type: Biochemistry and molecular biology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Arunachalam J, India; Bharara T, India

Received: August 28, 2022
Peer-review started: August 28, 2022
First decision: November 30, 2022
Revised: December 8, 2022
Accepted: February 2, 2023
Article in press: February 2, 2023
Published online: March 27, 2023

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Abstract

BACKGROUND

Understanding the humoral response pattern of coronavirus disease 2019 (COVID-19) is one of the essential factors to better characterize the immune memory of patients, which allows understanding the temporality of reinfection, provides answers about the efficacy and durability of protection against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and consequently helps in global public health and vaccination strategy. Among the patients who became infected with SARS-CoV-2, the majority who did not progress to death were those who developed the mild COVID-19, so understanding the pattern and temporality of the antibody response of these patients is certainly relevant.

AIM

To investigate the temporal pattern of humoral response of specific immunoglobulin G (IgG) in mild cases of COVID-19.

METHODS

Blood samples from 191 COVID-19 real-time reverse transcriptase-polymerase chain reaction (RT-qPCR)-positive volunteers from the municipality of Toledo/Paraná/Brazil, underwent two distinct serological tests, enzyme-linked immunosorbent assay, and detection of anti-nucleocapsid IgG. Blood samples and clinicoepidemiological data of the volunteers were collected between November 2020 and February 2021. All assays were performed in duplicate and the manufac-

turers' recommendations were strictly followed. The data were statistically analyzed using multiple logistic regression; the variables were selected by applying the $P < 0.05$ criterion.

RESULTS

Serological tests to detect specific IgG were performed on serum samples from volunteers who were diagnosed as being positive by RT-qPCR for COVID-19 or had disease onset in the time interval from less than 1 mo to 7 mo. The time periods when the highest number of participants with detectable IgG was observed were 1, 2 and 3 mo. It was observed that 9.42% of participants no longer had detectable IgG antibodies 1 mo only after being infected with SARS-CoV-2 and 1.57% were also IgG negative at less than 1 mo. At 5 mo, 3.14% of volunteers were IgG negative, and at 6 or 7 mo, 1 volunteer (0.52%) had no detectable IgG. During the period between diagnosis by RT-qPCR/symptoms onset and the date of collection for the study, no statistical significance was observed for any association analyzed. Moreover, considering the age category between 31 and 59 years as the exposed group, the *P* value was 0.11 for the category 31 to 59 years and 0.32 for the category 60 years or older, showing that in both age categories there was no association between the pair of variables analyzed. Regarding chronic disease, the exposure group consisted of the participants without any comorbidity, so the *P* value of 0.07 for the category of those with at least one chronic disease showed no association between the two variables.

CONCLUSION

A temporal pattern of IgG response was not observed, but it is suggested that immunological memory is weak and there is no association between IgG production and age or chronic disease in mild COVID-19.

Key Words: Humoral response; Immunoglobulin G antibody; Immune memory; Mild cases COVID-19; SARS-CoV-2 infection; Serological test

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Core Tip: This study suggests that no precise temporal pattern of humoral immunoglobulin G (IgG) response could be established. This fact suggests the absence of a robust immunological memory in mild cases of coronavirus 2019 disease, and furthermore, due to the lack of association between IgG response and age group, in mild cases of the disease the elderly do not appear to be a risk group for infection.

Citation: Pilati Campos IM, Marques M, Peiter GC, Brandalize APC, dos Santos MB, de Melo FF, Teixeira KN. Temporal pattern of humoral immune response in mild cases of COVID-19. *World J Biol Chem* 2023; 14(2): 40-51
URL: https://www.wjgnet.com/1949-8454/full/v14/i2/40.htm
DOI: https://dx.doi.org/10.4331/wjbc.v14.i2.40

INTRODUCTION

Coronavirus disease 2019 (COVID-19), a disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan, China, in December 2019 and spread worldwidevery quickly, causing an unprecedented pandemic that impacted healthcare systems, the economy, politics, and social organization. Patients with COVID-19 may be asymptomatic or present with critical illness, and with symptoms including fever, cough, sore throat, malaise and myalgia. Some patients may experience gastrointestinal symptoms such as anorexia, nausea, and diarrhea[1].

Asymptomatic patients can be assumed to be uninfected, and thus they can be the focus of new outbreaks of the infection by transmitting the virus to healthcare workers or individuals with risk factors[1,2]. According to some studies, risk factors for complications of COVID-19 include advanced age, cardiovascular disease, chronic lung disease, diabetes, obesity and immunosuppression[3-7].

Therefore, the influence of comorbidities on the immune response profile and disease susceptibility has been widely discussed. The main comorbidities involved in this study are diseases of the immune system, such as asthma, rheumatoid arthritis (RA) and autoimmune gastritis (AIG). Although patients with severe asthma have been associated with a higher risk of COVID-19-related death[8], studies have indicated that the disease was not statistically associated with increased risk of infection or being hospitalized due to the disease[9,10].

Regarding the gastrointestinal system diseases, specifically AIG, the data in the literature indicate no relationship between autoimmunity and increased susceptibility to SARS-CoV-2[11]. On the other hand,

in relation to RA, a study of patients with rheumatic diseases (91.89% were autoimmune with 72.97% of RA, and 18.92% of systemic lupus erythematosus) showed that cardiovascular manifestations caused by COVID-19 appeared more frequently in patients with rheumatic diseases[12].

According to the Chinese data, 81% of individuals infected with SARS-CoV-2 developed mild or moderate COVID-19, 14% developed severe disease, and only 5% developed critical disease[13]. Therefore, it is critical to study asymptomatic cases and those with mild symptoms, as they represent a risk of COVID-19 spreading. One example is the infection of 71 individuals originated from an asymptomatic case in China[14]. Furthermore, it has been evidenced that mild cases of COVID-19 also cause an increase in the need for primary health care, potentially lasting up to 3 mo[15], which can further burden the health care system.

Another point that emphasizes the importance of studying mild cases is the sequelae left by the virus. SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) as a functional receptor to invade host cells. This enzyme's main function is the regulation of angiotensin 2 and is highly expressed in the lungs, intestine and kidneys[16]. This gives the pathogen a high capacity of dissemination[16,17]. Thus, although the virus mainly infects the respiratory system, its systemic dissemination can occur, affecting several organs[17].

As respiratory sequelae, besides lung damage, the main consequences observed were alterations in taste and smell, such as anosmia, hyposmia, ageusia or dysgeusia[17,18]. Such symptoms were mainly associated with the appearance of symptomatic manifestations of COVID-19, since the gateway of the virus into the body are both the oral and nasal cavities, the sites whose epithelial tissue presents receptors for these senses[17].

Furthermore, one study demonstrated the high presence of ACE2 in biopsies of the olfactory mucosa, and the enzyme is mainly present in Bowman's glands[16]. In addition, other reported sequelae involve the cardiovascular system, such as heart attack and pulmonary thromboembolism, as well as kidney disease, liver damage, and even neurological changes[17].

As for the differences in immune response between mild and severe cases of COVID-19, it was observed that patients with critical illnesses showed a higher and earlier immunoglobulin G (IgG) and immunoglobulin A (IgA) response against SARS-CoV-2, as well as high viral neutralization. On the other hand, 75% of patients with mild symptoms developed antibodies and these showed low or even no viral neutralization rate[19,20].

Accordingly, this study set out to evaluate the temporal pattern of IgG anti-SARS-CoV-2 response in mild cases of COVID-19. To this end, the study analyzed data from patients presenting with the disease between September 2020 and January 2021, prior to the introduction of vaccines.

MATERIALS AND METHODS

Population study and data collection

This study was approved by the Ethics Committee for research with humans of the Setor de Ciências da Saúde-Universidade Federal do Paraná (UFPR)/Brazil (Protocol no. 35872520.8.0000.0102) and all participants signed an informed consent form. Volunteers older than 18 years (n=393) were tested for COVID-19 by real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) and 191 were positive. Blood samples and clinicoepidemiological data from the volunteers were collected from November 2020 to February 2021. The clinicoepidemiological data were collected by means of a semi-structured questionnaire. Venous blood was collected from each volunteer in a tube without anticoagulant and the serum was separated by centrifugation at 2000 rpm for 2 min. At this time, COVID-19 vaccines were not yet being applied in Brazil. The 191 individuals diagnosed as being positive for COVID-19 by RT-qPCR were included in this study; all were residents of the municipality of Toledo/Paraná/Brazil. Serum samples were subjected to two different serological tests by enzyme-linked immunosorbent assay, a commercial test-Allserum EIA COVID19 IgG (MBiolog Diagnostics) and a test developed by UFPR/Setor Litoral[21]. Both tests detected anti-nucleocapsid IgG, the secondary antibody was bound to Horseradish peroxidase, chromogenic substrate was Tetramethylbenzidine and absorbance reading was performed at 450 nm. All assays were performed in duplicate and the manufacturers' recommendations were strictly followed; assays were analyzed by UV/Vis Multiskan Sky spectrometer (Thermo Fisher Scientific Inc.).

Statistical analysis

A database containing clinicoepidemiological characteristics and serological results was prepared. To perform multiple logistic regression, variables were selected by applying the $P < 0.05$ adjusted odds ratio criterion and using the Maximum likelihood estimation. The final model was obtained after testing for all possible multiple interactions with subsequent verification of model fit by the Hosmer & Lemeshow method. A receiver operating characteristic (ROC) curve was done to evaluate the ability of the model created to represent reality.

RESULTS

Serum samples from the volunteers spanned a time interval of less than 1 mo up to 7 mo between positive diagnosis for COVID-19/symptoms onset and serological testing for specific IgG. The majority of study participants (62.83%) had detectable IgG for SARS-CoV-2, of which 37.17% had no antibodies at the time of blood collection. Furthermore, as shown in Figure 1, regarding the residence time of IgG detectable by serological testing, only 1 volunteer (0.52%) was IgG positive for 7 mo. The time periods where the highest number of participants with detectable IgG was observed were 1, 2 and 3 mo, with 46 (24%), 25 (13.08%) and 19 (9.94%), respectively. Twelve participants (6.28%) had IgG antibodies at the end of 4 mo, 4 participants (2.09%) remained IgG positive at the end of 5 mo, 8 (4.19%) had IgG for 6 mo, and 5 (2.61%) were IgG positive for less than 1 mo.

Figure 1 also shows that 18 individuals (9.42%) no longer had detectable anti-SARS-CoV-2 IgG antibodies one month after being infected with SARS-CoV-2 and 3 (1.57%) were negative at less than 1 mo. Seventeen volunteers (8.90%) had no detectable anti-SARS-CoV-2 IgG after 2 mo; the same was observed with 16 volunteers (8.37%) at the end of 3 mo and 9 participants (4.71%) at the end of 4 mo. At 5 mo, 6 volunteers (3.14%) had negative anti-SARS-CoV-2 IgG, and at 6 or 7 mo 1 volunteer (0.52%) had no detectable IgG.

As shown in Table 1, regarding the period of time elapsed between diagnosis by RT-qPCR/symptoms onset and the date of blood collection for the study, no statistical significance was observed between the variables.

Regarding age, most participants (115 or 60.21%) were between 31 and 59 years old; 57 (29.84%) were between 18 and 30 years old, and 19 (9.95%) participants were 60 years old or older. Considering the age group between 31 and 59 years as the exposed group, the *P* value was 0.11 for the age group of 31 - 59 years and 0.32 for the group of 60 years or older, showing that there was no association between the pair of variables analyzed.

About the presence of chronic diseases, 135 (70.68%) participants had no comorbidity. On the other hand, 45 (23.56%) individuals had one chronic disease, while 10 (5.24%) had two and one (0.52%) participant had three. Regarding this variable, the exposure group considered was the participants without any comorbidity, so that the *P* value of 0.07 for those with at least one chronic disease showed no association between the two variables studied.

The statistical model showed adequate fit evaluated by the Hosmer & Lemeshow method ($\chi^2 = 3.656$; GL = 8; $P = 0.887$). The area under the ROC curve showed that the estimated probability model could predict approximately 67.59% of the factors associated with the outcome.

DISCUSSION

The viral proteins play a striking role in diagnosing COVID-19 and monitoring the production of antibodies against the coronavirus by serological tests. In this regard, tests for COVID-19 can be either nucleic acid amplification tests (RT-qPCR) or serological tests. While RT-qPCR is recommended for active coronavirus infection, serological tests are recommended for antibody response [immunoglobulin M (IgM) and IgG][22]. Thus, serologic testing and antibody analysis are useful to verify whether there has been previous exposure to the virus and to quatify the patient's humoral immunity levels and types of antibodies produced[23].

Therefore, the production of specific antibodies is essential, since they are responsible for effective protection against the severe forms of the disease, even though there are other cells, such as TCD^{4+} and TCD^{8+}, that act in the immunity process[24]. In addition, IgM antibodies provide the first line of defense during infections, while IgG production provides immunity and long-term memory[25].

The level of antibody production depends on the elapsed time of infection, the severity of the disease, the viral load to which the patient has been exposed, and individual patient characteristics such as age, sex, and pathogen elimination[26]. There may also be variations in the detection of antibody levels according to the sensitivity of the serological test used.

Regarding the elapsed time of infection, while IgM antibodies can be detected at about five days of infection and reach higher rates between two and three weeks of illness, IgG antibodies begin to be produced about 14 d after the symptoms onset and individuals with more severe disease have higher antibody levels[27,28]. Regarding COVID-19, the observation period for most studies on the production of specific anti-SARS-CoV-2 antibodies is 12 wk, and it is still unclear how antibody titers may change in subsequent periods[29].

In a study conducted in Wuhan, China, after confirmation of coronavirus infection by RT-qPCR, asymptomatic individuals were recruited to detect levels of anti-SARS-CoV-2 antibodies. Of a total of 63 individuals with asymptomatic infections, 38.1% (24 patients) produced no antibodies and 61.9% (39 patients) produced only small titers. Six (11.8%) out of 51 patients with mild symptoms, produced no antibodies and 88.2% (45 patients) produced higher levels of antibodies when compared to asymptomatic patients.

Table 1 Temporal pattern of immunoglobulin G and baseline characteristics of real-time reverse transcriptase-polymerase chain reaction positive individuals for coronavirus disease 2019

Variable		*n*	%	*P* value	OR adjusted	95%CI
IgG						
	Negative	71	37.17			
	Positive	120	62.83			
Age						
	18 to 30 yr	57	29.84			
	31 to 59 yr	115	60.21	0.11	1.26	(0.89-3.48)
	60 yr or +	19	9.95	0.32	1.92	(0.60-6.60)
Time						
	< 1 mo	8	4.19	0.09	0.79	(0.16-3.83)
	1 mo	64	33.51			
	2 mo	42	21.99	0.18	0.56	(0.24-1.30)
	3 mo	35	18.32	0.13	0.50	(0.20-1.22)
	4 mo	21	10.99	0.36	0.61	(0.21-1.75)
	5 mo	10	5.24	0.09	0.29	(0.07-1.21)
	6 mo	9	4.71	0.25	3.55	(0.41-30.69)
	7 mo	2	1.05	0.39	0.27	(0.01-5.14)
Chronic disease						
	Not	135	70.68			
	Yes	56	29.32	0.07	2.03	(0.94-4.40)
Number of chronic diseases						
	0	135	70.68			
	1	45	23.56			
	2	10	5.24			
	3	1	0.52			

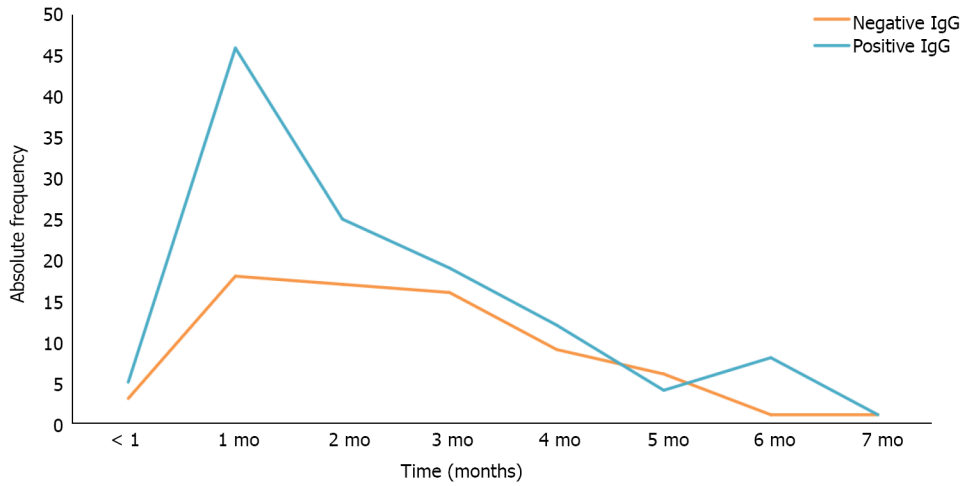
IgG: Immunoglobulin G; CI: Confidence interval; OR: Odds ratio.

In the same study, in asymptomatic individuals, antibody production started seven days after exposure, peaked between 10 and 25 d, and decreased rapidly thereafter. On the other hand, in individuals with mild symptoms, one day after the onset of symptoms, antibodies were already produced, even at a low level, and titration increased persistently up to 22 d, maintaining high levels for at least 65 d[30].

In a study of 164 participants in Singapore, 19 patients (12%) did not develop neutralizing antibodies against SARS-CoV-2; 44 patients (27%) produced antibodies early (approximately 20 d after symptoms onset), but disappeared in less than 180 d; 46 patients (28%) had neutralizing antibodies for more than 180 d after symptoms onset; 52 patients (32%) had minimal decay of neutralizing antibodies; and three patients (2%) who had increased neutralizing antibodies 90 d after symptoms onset[31].

Similarly, 140 patients with COVID-19 positive for RT-qPCR were recruited for a study in France, of whom 44 were admitted to the intensive care unit (ICU), 42 were hospitalized without the need for ICU, and 54 received outpatient treatment only (including eight asymptomatic cases). It was observed that most patients in the different groups produced neutralizing antibodies, but the neutralizing activity was variable, *i.e.* higher in the group of patients admitted to the ICU, so that only one patient in this group did not develop a neutralizing antibody response at the time of collection. In contrast, 21.9% of hospitalized patients and 25% of outpatients treated did not develop neutralizing antibodies at the time of the study[32].

This study supports the hypothesis that seroconversion is observed more frequently in individuals with severe symptoms and that they have higher antibody titers than mild and asymptomatic cases. This means that plasma titers are approximately eight times higher for severe cases[33].



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Figure 1 Correlation between positive or negative immunoglobulin G in volunteers and the time after diagnosis of coronavirus disease 2019/symptoms onset. IgG: Immunoglobulin G.

Therefore, this study focused on understanding humoral immunity in mild cases of COVID-19 and regarding the time period of IgG detection in the serum of patients, similar results were obtained to previous studies. This finding contributes and adds data to the current knowledge about the humoral response against mild COVID-19 as it is a study conducted in a Western country with a larger sample size.

However, the temporal profile of the antibody response raised a concern by other authors[34-36]. Humoral immunity against SARS-CoV-2 does not appear to be durable, especially in individuals with mild symptoms or those who are asymptomatic, which make up the majority of COVID-19 cases. This fact is corroborated by the several doses of the SARS-CoV-2 vaccine, which is currently in its fourth dose in Brazil. On the other hand, it was also found that individuals with low titers or even undetectable levels of neutralizing antibodies can still be protected from subsequent infections, considering that memory B cells are still present in recovered patients[37,38].

The effect of age on immunity against SARS-CoV-2 has been widely discussed. A cohort study developed by the University of Virginia analyzed the antibody responses in individuals who received two doses of the vaccine BNT162b2 (Pfizer®) or mRNA-1273 (Moderna®) and had a blood sample collected seven to 31 d after the second dose. The results showed that participants aged 50 years and older who received BNT162b2 had lower pre-boost IgG levels than participants younger than 50 years who received the same vaccine. Individuals aged 50 years and older who received BNT162b2 had post-boost IgG levels that were also lower than levels found in younger participants[39].

Another study examined the immune response in elderly participants and younger healthcare professionals following immunization with the BNT162b. The results showed that after the first dose of the vaccine, IgG or IgA levels were lower in older individuals. In addition, elderly participants showed lower interferon-γ and interleukin (IL)-2 production by T cells specific against SARS-CoV-2 when compared to younger individuals[40].

Furthermore, a cohort study conducted in Greece analyzed the IgG response against the protein S of SARS-CoV-2 in a group of individuals after immunization with two doses of the BNT162b2 vaccine. The results revealed that younger patients (21-30 years old) had the highest antibody levels in both periods [41].

On the other hand, some studies have observed that older patients have been related to higher levels of antibodies against SARS-CoV-2. A study from Union Hospital (Huazhong University of Science and Technology, Wuhan, China) conducted with convalescent patients identified the presence of anti-SARS-CoV-2 antibodies one year after infection, in addition to a difference in IgG response according to the age of the patients. It was observed that the mean IgG antibody level was relatively low in younger convalescent patients (aged 21 - 35 years), and gradually increased to about 60% in older patients. The mean anti-SARS-CoV-2 IgG level was significantly higher in patients older than 35 years when compared to those younger than or equal to 35 years[42]. In our study, however, we found no statistical significance in the association between age and anti-SARS-CoV-2 IgG antibody levels. Seroconversion in the group of older individuals was not lower when compared to younger participants, demonstrating that age is possibly not a risk factor when analyzing mild cases of COVID-19.

Regarding the association between COVID-19 and pre-existing comorbidities, those with the highest association are hypertension and obesity, followed by metabolic disease, cardiovascular disease, neurological disease, chronic lung disease, kidney disease, asthma, immunosuppression, gastrointestinal or liver disease, and finally autoimmune disease[43]. According to some authors, COVID-19

has also been associated with type 2 diabetes mellitus (DM2), cancer, and chronic kidney disease[44].

It has been found in numerous studies that individuals with DM2 may have higher severity and mortality from COVID-19. This fact is due to the existing inflammatory condition in these patients, with higher levels of pro-inflammatory molecules, such as cytokines, especially IL-6. In addition, the presence of DM2 causes the response to SARS-CoV-2 to show a large amount of interferon and a delayed Th1 and Th17 response, which contributes to a more intense inflammatory response[45]. An example of this is a study showing that increased viral replication and production of pro-inflammatory cytokines may be related to high glucose concentration[46].

Coronaviruses bind to ACE2, reducing the activity of this receptor and increasing vascular permeability[47]. However, in individuals with systemic arterial hypertension (SAH) and DM, there is a higher number of these receptors when compared to the general population, which may explain the more severe cases of COVID-19 in these patients. Furthermore, SARS-CoV-2 produces endothelial injury, causing an inflammatory vascular state, pro-coagulant state, and a cellular infiltrate, also clarifying the more severe symptoms in individuals with these chronic diseases[48,49].

In addition, the presence of SAH also determines a pro-inflammatory state, arising from the endothelial dysfunction caused by this disease, leading to excessive activation of coagulation and platelets, in addition to the production of cytokines, antimicrobial peptides, and reactive oxygen species. This excessive activation may not only cause damage to the respiratory epithelium, but also reduce lung function and increase the local inflammatory response, contributing to further occurrence of complications from COVID-19[50,51].

An important question of the study is how COVID-19 affects patients with autoimmune diseases, such as RA. In a study of 11 122 individuals with COVID-19, patients with RA were found to have a higher chance of hospitalization or death than healthy individuals, and the study used an unadjusted model. However, when adjusting for age, sex, and comorbidities, no greater chance of unfavorable outcomes was observed[51]. Thus, AR is associated with a higher risk of infection and death in patients with COVID-19 when taking into account active AR, the presence of other diseases and the use of medications such as Rituximab, sulfasalazine or other immunosuppressive drugs[52].

Regarding the association between AIG and COVID-19, a study conducted at the Foundation of San Matteo Hospital, Italy, analyzed the susceptibility to COVID-19 in 400 drug-free immunosuppressive patients with autoimmune diseases, 100 of whom had AIG. The findings showed that among the individuals with AIG, seven (7%) had already tested positive for COVID-19, one (1%) required hospitalization for COVID-19, and 43 (43%) were vaccinated for SARS-CoV-2.

Furthermore, considering all investigated autoimmune diseases, molecular nasopharyngeal swabs and/or serology for SARS-CoV-2 testing showed that 33 (8.2%) tested positive[53]. These data are similar to those reported in the general population in the same geographical area in Italy[53], suggesting that the risk of COVID-19 in individuals with autoimmune diseases appears to be the same as in the general population.

Asthma is still being studied as a risk factor for COVID-19. The proposed hypothesis that the occurrence of more severe complications caused by COVID-19 in patients with asthma is due to a possible interaction between the pathobiology of SARS-CoV-2 and asthma. Thus, since the virus causes an intense inflammatory response and asthmatic individuals already have narrowed airways with high mucus production, pneumonia caused by the virus can lead to severe complications[54]. However, asthma can also lead to favorable outcomes, since it induces a negative regulation of ACE2, an enzyme that assists in the process of viral entry of SARS-CoV-2 into lung tissue[55]. On the other hand, a meta-analysis study did not identify a statistically significant increase in mortality and a worse prognosis for COVI-19 in asthmatic individuals[9].

Thus, although there are studies indicating the need for more intensive treatment in individuals with DM2, SAH, and other comorbidities[56], in patients with mild symptoms, this association does not seem to materialize, as the presence of previous diseases was not statistically significant for seroconversion in our study participants.

CONCLUSION

This study suggests that in mild cases of COVID-19, it is not possible to establish a temporal relationship of specific IgG production, raising the hypothesis that such a relationship may, in fact, not exist or that perhaps there is more than one type of relationship since there is interference from several factors, such as age, sex, presence of comorbidities, viral elimination, viral load, among others. It is also suggested that the virus generates a weak and non-lasting immune response in mild cases. Furthermore, a lower production of IgG antibodies was not observed in the elderly and in individuals with previous chronic diseases, leading to the conclusion that in mild cases of COVID-19, these patients may not be a risk group for unfavorable outcomes when analyzing the humoral response.

ARTICLE HIGHLIGHTS

Research background

The molecular test used in the diagnosis of coronavirus disease 2019 is very specific and sensitive, however, it is not able to detect previous exposure to the virus nor to assess immunological memory. Therefore, serological tests that have this capability are used as tools for understanding the course of the humoral immune response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

Research motivation

The motivation for this study arose from the serological test developed at the Federal University of Paraná, which in the validation process showed better sensitivity than commercial tests. A more sensitive test allows specific antibodies to be detected even at low titers, and thus to effectively assess whether there is still a protective antibody response in individuals who have been infected by the virus.

Research objectives

The aim of this study was to identify if a pattern of SARS-CoV-2 specific immunoglobulin G (IgG) production can be determined according to the time elapsed since diagnosis of the disease/onset of symptoms. The data could indicate, for example, the interval between vaccination doses.

Research methods

This study was initiated after approval by the ethics committee. The participants were tested by real-time reverse transcriptase-polymerase chain reaction, the municipal government provided us with the data. Only positive cases were included in the study. Blood collection was performed by our research team and the method used for specific IgG antibodies was the indirect enzyme-linked immunosorbent assay. Statistical analyses were performed by the statistician of the research group, one of the authors of the manuscript.

Research results

The results of the study showed that there is no time pattern for the production of specific IgG. Less than one month after infection, some participants no longer have detectable IgG in the serum, while others have the antibodies seven months after infection.

Research conclusions

In addition to the impossibility of establishing a temporal pattern of IgG response, the data indicate that SARS-CoV-2 does not appear to induce a long-lasting humoral response.

Research perspectives

The study perspective is to analyze the immunoglobulin M (IgM) response of the same volunteers and determine the titers of both IgG and IgM to better understand seroconversion and the robustness of the anti-SARS-CoV-2 antibody response.

ACKNOWLEDGEMENTS

The authors would like to thank the Universidade Federal do Paraná, Municipal Health Secretariat of Toledo/Paraná/Brazil and Professor Ph.D. Luciano Fernandes Huergo for support.

FOOTNOTES

Author contributions: Teixeira KN and Brandalize APC designed, coordinated the study and interpreted the data; Pilati Campos IM and Peiter GC carried out the experiments, acquired and analyzed data; dos Santo MB carried out the statistical analyses; Pilati Campos IM and Marques M reviewed the literature and wrote the manuscript; Teixeira KN and de Melo FF reviewed the manuscript.

Institutional review board statement: The study was approved by the Ethics Committee for research with humans of the Setor de Ciências da Saúde-Universidade Federal do Paraná (UFPR)/Brazil (Protocol no. 35872520.8.0000.0102).

Informed consent statement: All study participants were over 18 years old, and they read and signed the informed consent statement.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The study participants gave informed consent for data disclosure in an anonymous way,

without exposing their identity.

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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S-Editor: Fan JR
L-Editor: Ma JY-MedE
P-Editor: Fan JR

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World J Biol Chem 2023 March 27; 14(2): 52-61

DOI: 10.4331/wjbc.v14.i2.52

ISSN 1949-8454 (online)

ORIGINAL ARTICLE

Observational Study

Correlation of serum SARS-CoV-2 IgM and IgG serology and clinical outcomes in COVID-19 patients: Experience from a tertiary care centre

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Specialty type: Immunology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Ait Addi R, Morocco; Hasan A, Egypt

Received: October 10, 2022
Peer-review started: October 10, 2022
First decision: January 3, 2023
Revised: January 12, 2023
Accepted: February 13, 2023
Article in press: February 13, 2023
Published online: March 27, 2023

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Abstract

BACKGROUND

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus has become a pandemic for the last 2 years. Inflammatory response to the virus leads to organ dysfunction and death. Predicting the severity of inflammatory response helps in managing critical patients using serology tests IgG and IgM.

AIM

To investigate the correlation of the serology (IgM and IgG) with reverse transcriptase polymerase chain reaction (RT-PCR) status, disease severity [mild to critical], intensive care unit (ICU) admission, septic shock, acute kidney injury, and in-hospital mortality.

METHODS

We conducted a longitudinal study to correlate serum SARS-CoV-2 immunoglobulin M (IgM) and immunoglobulin G (IgG) serology with clinical outcomes in coronavirus disease 2019 (COVID-19) patients. We analyzed patient data from March to December 2020 for those who were admitted at All India Institute of Medical Sciences Rishikesh. Clinical and laboratory data of these patients were collected from the e-hospital portal and analyzed. A correlation was seen with clinical outcomes and was assessed using MS Excel 2010 and SPSS software.

RESULTS

Out of 494 patients, the mean age of patients was 48.95 ± 16.40 years and there were more male patients in the study (66.0%). The patients were classified as mild-moderate 328 (67.1%), severe 131 (26.8%), and critical 30 (6.1%). The mean duration from symptom onset to serology testing was 19.87 ± 30.53 d. In-hospital mortality was observed in 25.1% of patients. The seropositivity rate (*i.e.*, either IgG or IgM > 10 AU) was 50%. IgM levels (AU/mL) (W = 33428.000, $P \leq 0.001$) and IgG levels (AU/mL) (W = 39256.500, $P \leq 0.001$), with the median IgM/ IgG levels (AU/mL), were highest in the RT-PCR-Positive group compared to RT-PCR-Negative clinical COVID-19. There was no significant difference between the two groups in terms of all other clinical outcomes (disease severity, septic shock, ICU admission, mechanical ventilation, and mortality).

CONCLUSION

The study showed that serology levels are high in RT-PCR positive group compared to clinical COVID-19. However, serology cannot be useful for the prediction of disease outcomes. The study also highlights the importance of doing serology at a particular time as antibody titers vary with the duration of the disease. In week intervals there was a significant correlation between clinical outcomes and serology on week 3.

Key Words: Inflammatory response; Reverse transcription polymerase chain reaction; SARS-CoV-2; Serology IgM and IgG

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Core Tip: Coronavirus disease 2019 (COVID-19) serology levels are high in reverse transcriptase polymerase chain reaction positive group compared to clinical COVID-19. However, serology cannot be useful for the prediction of disease outcomes. The study also highlights the importance of doing serology at a particular time as antibody titres vary with the duration of the disease. In week interval there were significant correlation with clinical outcomes and serology on week 3.

Citation: Suresh M, Kumar P, Panda PK, Jain V, Raina R, Saha S, Vivekanandhan S, Omar BJ. Correlation of serum SARS-CoV-2 IgM and IgG serology and clinical outcomes in COVID-19 patients: Experience from a tertiary care centre. *World J Biol Chem* 2023; 14(2): 52-61
URL: https://www.wjgnet.com/1949-8454/full/v14/i2/52.htm
DOI: https://dx.doi.org/10.4331/wjbc.v14.i2.52

INTRODUCTION

Coronavirus disease 2019 (COVID-19) has affected almost 581 million people with around 6.4 million deaths as of July 2022 [World Health Organization (WHO)][1]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can infect individuals from different age groups and causes a wide spectrum of disease manifestations ranging from asymptomatic, to mild, moderate to severe symptoms with possible fatal outcomes[2]. Age, sex, pre-existing comorbidities, host genetics as well as host immune response are the key factors determining the outcomes[3]. The reverse transcriptase polymerase chain reaction (RT-PCR) assay is the right method to diagnose SARS-CoV-2. Unfortunately, the sensitivity of the RNA test in the real world is not satisfactory and, false-negative and false-positive cases have also been reported owing to several factors[4]. According to recent WHO case definitions, the RT-PCR negative patients who meet clinical and epidemiological criteria or patients with severe acute respiratory illness who have typical chest imaging features or unexplained anosmia or ageusia are termed as probable COVID-19 patients, better term would be RT-PCR-negative clinical COVID-19[5,6].

Serological tests are increasingly applied for the diagnosis of SARS-CoV-2 infection, though not evidenced by various guidelines. Blood levels of immunoglobulin SARS-CoV-2 immunoglobulin G (IgG) & immunoglobulin M (IgM) are also deployed for evaluating immune responses and confirming the diagnosis in symptomatic patients presenting outside the window of positivity for RT-PCR-based SARS-CoV-2 testing[7]. Few studies have assessed the utility of seroconversion profiles to predict infection severity or outcomes following SARS-CoV-2 infection. A strong association was observed between the magnitude of antibody response and patient survival, disease severity, and fatal outcomes [8]. Furthermore, several studies have documented discrepancies in findings related to the timing of SARS-CoV-2 antibody seroconversion and the onset of symptoms[9-11]. More information about the dynamics of the early humoral immune response is needed to realize the full potential of serological

testing for SARS-CoV-2. The dynamics of antibody responses, in COVID-19 patients with different clinical presentations, are still not well-characterized. Such information can help our understanding of the nature of COVID-19 infection and guide patient management.

Here, we studied the seropositivity and kinetics of SARS-CoV-2 IgM and IgG antibodies in blood samples collected between 2 to 85 d post-symptoms onset from a cohort of 493 COVID-19 patients. The objectivity was the correlation of the serology (IgM and IgG) with RT-PCR status, disease severity (mild to critical), intensive care unit (ICU) admission, septic shock, acute kidney injury (AKI), and in-hospital mortality.

MATERIALS AND METHODS

Study design and setting

The study was an observational longitudinal study conducted on COVID-19 patients admitted to a tertiary care hospital, All India Institute of Medical Sciences (AIIMS), Rishikesh, India from August 2020 to November 2020. The study was designed according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guidelines.

Inclusion criteria

COVID-19 patients with detectable SARS-CoV-2 RNA in respiratory samples since disease onset. Clinical COVID-19 patients i.e. cases with clinical manifestations characteristic of COVID-19 but with negative SARS-CoV-2 RT-PCR test from admission until discharge[1,2]. Patients of both genders with age ≥ 15 years. Patients with complete data on serological results available in files.

Exclusion criteria

Patients not fulfilling COVID-19 diagnostic criteria as per institutional protocol. Asymptomatic patients, pregnant women, and patients having incomplete data.

Case definitions

COVID-19 Severity classification: Patients were classified as mild, moderate, severe, and critical according to the WHO guidelines[1].

Serological tests

iFlash-SARS-CoV-2 (Shenzhen Yhlo Biotech Co. Ltd.), a paramagnetic particle-based chemiluminescent immunoassay (CLIA) was used for the determination of IgM and IgG antibodies against SARS-CoV-2 nucleocapsid protein and spike protein. According to the manufacturer's inserts [V1.0 English Fd. 2020–02-20], the IgM and IgG cut-off is 10 AU/mL, *i.e.*, an antibody titer above titer over 10 AU/mL was regarded as positive.

Treatment of patients

Patients were treated uniformly as per institutional guidelines.

Participants' enrolment

All COVID-19 admitted patients at All India Institute of Medical Sciences, Rishikesh during the above period.

Variables and outcome and data collection

Full information regarding demographic characteristics, the time course of symptoms, time of presentation and testing, presenting symptoms, final diagnosis, treatments received [*i.e.* oxygen therapy, corticosteroids, ICU admission, invasive ventilation requirement, and dialysis] were collected in master excel. The medical records were further critically reviewed for important missed data.

Study size

All consecutive patients during the above period.

Ethics

The Approval for this study was obtained from the institute ethics committee of AIIMS Rishikesh with approval no CTRI/2020/08/027169.

Statistical methods

All the statistical analyses were performed using the statistical package for social sciences (SPSS), Windows version 23 software package (SPSS, CHICAGO, IL, United States). Non-normally distributed continuous variables were presented as medians [interquartile ranges (IQR)]. Differences between non-

normally distributed continuous variables were assessed using the Mann–Whitney U test. Categorical variables were presented as counts (%). Differences between categorical variables were assessed using the χ^2 or Fisher's exact tests. A two-sided value of $P < 0.05$ was considered statistically significant.

Bias

As all patients sampling for IgG and IgM was conducted only once, and time to sampling may be an important variable that can confound the study results, we analyzed the association between different clinical outcomes and its association with IgG and IgM levels in a time-dependent manner based on the time interval between symptom onset and IgM and IgG testing. We used Bayesian latent class modeling for the evaluation of the diagnostic performance of RT-PCR, IgM, and IgG tests in COVID-19.

RESULTS

Demographic characteristics

A total of 494 hospitalized patients were enrolled in the study, among them 199 were RT-PCR positive and 294 were clinically diagnosed COVID-19 patients (Table 1, Figures 1 and 2).

Seropositivity status among COVID-19 patients

In this cohort of 494 data on seropositivity was available for 455 patients, and the seropositivity rate (*i.e.* either IgM or IgG > 10 AU) was 247 (54%). Out of these IgM seropositivity was observed in 103/455 (22.63%) and for IgG 224/455 (49.01%). IgM or IgG seropositivity increased to a peak at week 4 and then decreases after 4 wk (> 28 d, Figure 3).

Association between COVID-19 serology and RT-PCR status

There was a significant difference between the 2 groups in terms of IgM levels (AU/mL) (W = 33428.000, $P \leq 0.001$) and IgG levels (AU/mL) (W = 39256.500, $P \leq 0.001$), with the median IgM/ IgG levels (AU/mL) being highest in the RT-PCR-Positive group. In all weeks, there was a significant difference between the 2 groups except for week 4 (22-28 Days) there was no significant difference in terms of IgM and IgG levels (AU/mL) (Figure 4).

Association between COVID-19 serology and disease severity

There was no significant difference between the groups in terms of IgM levels (AU/mL) ($\chi^2 = 2.975$, $P = 0.395$) and IgG levels ($\chi^2 = 2.463$, $P = 0.482$). In week 3, there was a significant difference between the groups in terms of IgM Levels (AU/mL) ($\chi^2 = 7.732$, $P = 0.021$) and IgG levels (AU/mL) ($\chi^2 = 7.707$, $P = 0.021$), with the median IgM and IgG levels (AU/mL) being highest in the critical group. In all the other weeks, there was a significant difference between the 2 groups in terms of IgM and IgG levels (AU/mL) (Supplementary Figure 1).

Association of COVID-19 serology with acute respiratory distress syndrome types and Oxygen requirement

There was a significant difference between the 4 groups in terms of IgM Levels (AU/mL) ($\chi^2 = 7.985$, $P = 0.046$) and IgG levels (AU/mL) ($\chi^2 = 8.501$, $P = 0.037$). The median IgM levels (AU/mL) were highest in the mild acute respiratory distress syndrome (ARDS) group and median IgG levels (AU/mL) were highest in the Moderate ARDS group.

In all weeks no significant difference between the groups in terms of IgM levels and IgG levels. However, in week 3 there was a significant difference between the 4 groups in terms of IgM levels (AU/mL) ($\chi^2 = 10.837$, $P = 0.013$) and IgG of IgG levels (AU/mL) ($\chi^2 = 9.682$, $P = 0.021$). The median IgM levels (AU/mL) were highest in the Mild ARDS group and the median IgG levels (AU/mL) were highest in the severe ARDS group.

There was a significant difference between the 3 groups in terms of IgM levels (AU/mL) ($\chi^2 = 6.795$, $P = 0.033$), with the median IgM levels (AU/mL) being highest in the Oxygen Therapy: < 6 L/min group. There was no significant difference between the groups in terms of IgG Levels (AU/mL) ($\chi^2 = 4.532$, $P = 0.104$).

There was a significant difference between the 3 groups in terms of IgM levels (AU/mL) in week 1 ($\chi^2 = 6.053$, $P = 0.048$), with the median IgM levels (AU/mL) being highest in the Oxygen Therapy: < 6 L/min group, week 2 ($\chi^2 = 6.392$, $P = 0.041$), with the median IgM levels (AU/mL) being highest in the Oxygen Therapy: > 6 L/min group and Week 3 ($\chi^2 = 6.283$, $P = 0.043$), with the median IgM levels (AU/mL) being highest in the Oxygen Therapy: < 6 L/min group. There was a significant difference between the 3 groups in terms of IgG levels (AU/mL) ($\chi^2 = 8.629$, $P = 0.013$), with the median IgG levels (AU/mL) being highest in the Oxygen Therapy: > 6 L/min group. In all other weeks no significant difference between the groups in terms of IgM levels and IgG levels (Supplementary Figure 2).

Table 1 Demographic table

Features	Median [Q1-Q3] or frequency [%]
Age (yr)	50.00 [36.00-61.00]
Gender	
Male	326 [66.0]
Female	168 [34.0]
IgG (AU/mL)	7.82 [0.63-57.07]
IgG	
< 10 AU/mL	231[50.99]
> 10 AU/mL	224 [49.01]
IgM (AU/mL)	0.96 [0.48-7.68]
IgM	
< 10 AU/mL	352 [77.37]
> 10 AU/mL	103 [22.8]
RT-PCR	
Positive	199 [40.4]
Negative	294 [59.6]
Onset-Testing Interval (d)	12.00 [7.00-21.00]

RT-PCR: Reverse transcriptase polymerase chain reaction.

Association of COVID-19 serology with Septic shock

There was no significant difference between the groups in terms of IgM Levels (AU/mL) (W = 1191.500, *P* = 0.168) and IgG levels (AU/mL) (W = 19537.500, *P* = 0.261).

In all weeks no significant difference between the groups in terms of IgM levels and IgG levels. However, there was a significant difference between the 2 groups in terms of IgM levels AU/mL (W = 1827.000, *P* = 0.035), with the median IgM levels (AU/mL) being highest in the no Septic Shock group. In week 3 IgG levels (AU/mL) (W = 317.000, *P* = 0.022), with the median IgG levels (AU/mL) being highest in the Septic Shock group and in > 4 wk (W = 366.000, *P* = 0.042), with the median IgG levels (AU/mL) being highest in the no Septic Shock group (Supplementary Figure 3).

Association of COVID-19 serology with the requirement of ICU admission

There was no significant difference between the groups in terms of IgM levels (AU/mL) (W = 23685.000, *P* = 0.668) and IgG (W = 25763.500, *P* = 0.157).

In all weeks no significant difference between the groups in terms of IgM levels and IgG levels. However, there was a significant difference between the 2 groups in terms of IgM levels (AU/mL) on week 3 (W = 403.500, *P* = 0.031) and IgG (W = 460.000, *P* = 0.038) with the median IgM levels (AU/mL) being highest in the group requiring ICU admission (Supplementary Figure 4).

Association of COVID-19 serology with the requirement of mechanical ventilation

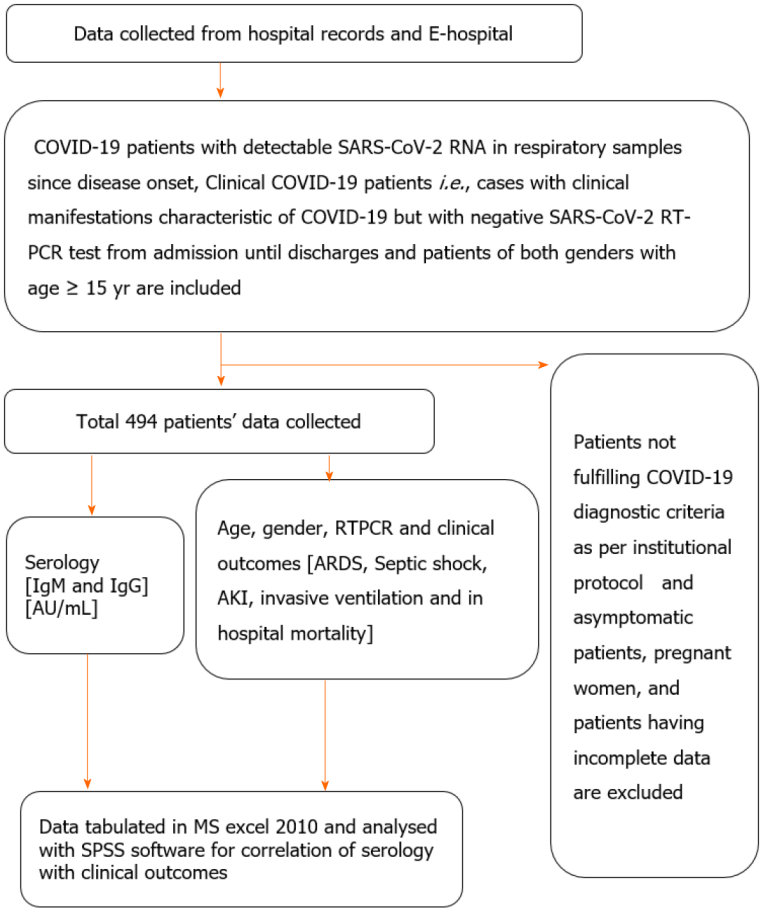
There was no significant difference between the groups in terms of IgM levels (AU/mL) (W = 20744.500, *P* = 0.099) and IgG levels (AU/mL) (W = 23067.000, *P* = 0.460).

In all weeks no significant difference between the groups in terms of IgM levels and IgG levels. However, there was a significant difference between the 2 groups in terms of IgM levels (AU/mL) on week 2 (W = 2070.000, *P* = 0.035) and > 4 wk (> 28 d) (W = 358.500, *P* = 0.033), with the median IgM levels (AU/mL) being highest in the no Invasive Ventilation group (Supplementary Figure 5).

Association of COVID-19 serology with AKI and requirement of dialysis

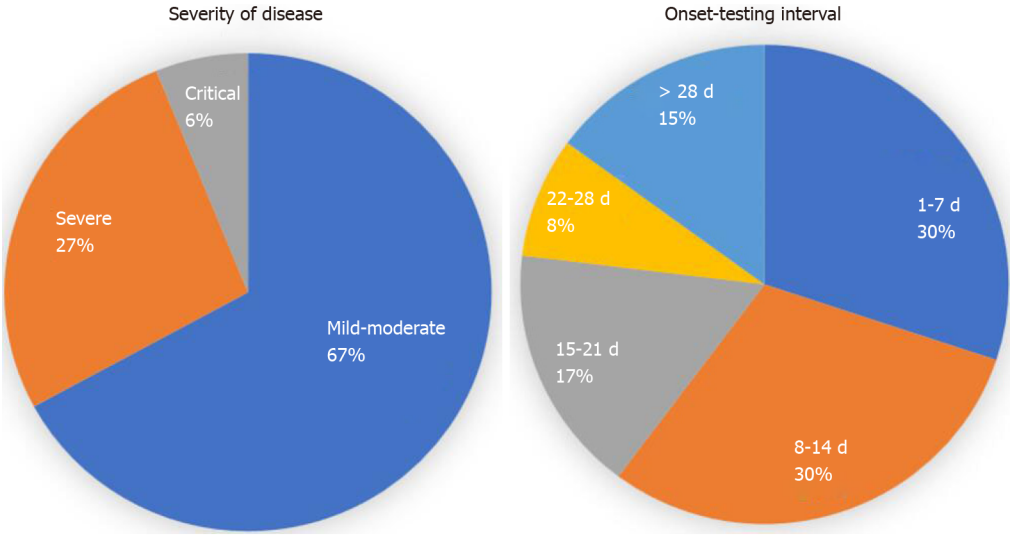
There was no significant difference between the groups in terms of IgM Levels (AU/mL) (W = 23261.500, *P* = 0.425) and IgG levels (AU/mL) (W = 26023.500, *P* = 0.767).

In all weeks no significant difference between the groups in terms of IgM levels and IgG levels. However, there was a significant difference between the 2 groups in terms of IgM levels (AU/mL) on week 2 (W = 2473.000, *P* = 0.008), and IgG levels (AU/mL) (W = 2755.500, *P* = 0.043) with the median IgM/ IgG levels (AU/mL) being highest in the no Acute Kidney Injury group.



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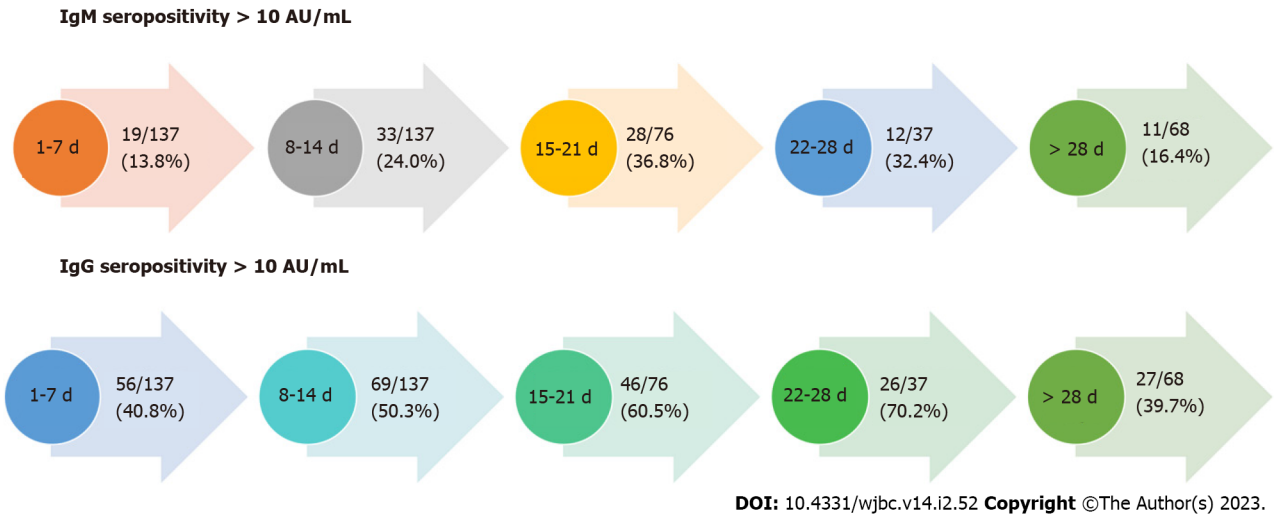
Figure 1 Study flow chart. AKI: Acute kidney injury; ARDS: Acute respiratory distress syndrome; COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; IgG: Immunoglobulin G; IgM: Immunoglobulin M; RT-PCR: Reverse transcriptase polymerase chain reaction.



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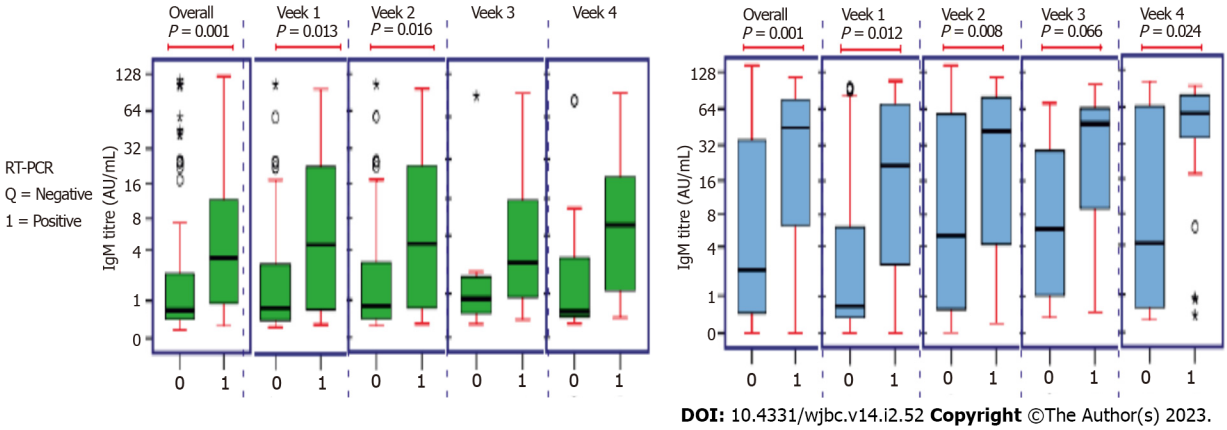
Figure 2 Baseline demographic characteristic of patients.

There was a significant difference between the 2 groups in terms of IgM levels (AU/mL) (W = 14962.000, $P \leq 0.001$), with the median IgM levels (AU/mL) being highest in the no Dialysis group. However, there was no significant difference between the groups in terms of IgG levels (AU/mL) (W = 14553.000, $P = 0.206$). In all weeks no significant difference between the groups in terms of IgM levels and IgG levels (Supplementary Figure 6).



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Figure 3 Seropositivity status with the duration of illness.



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Figure 4 Association of coronavirus disease 2019 serology and reverse transcriptase polymerase chain reaction status.

Association between COVID-19 serology and outcome: Survivor vs non-survivor

There was no significant difference between the groups in terms of IgM levels (AU/mL) (W = 21870.000, P = 0.058) and IgG levels (AU/mL) (W = 23088.500, P = 0.738).

In all the weeks there was no significant difference between the groups in terms of IgM levels and IgG levels. However, there was a significant difference between the 2 groups in terms of IgM levels (AU/mL) on week 4 (W = 136.500, P = 0.032) and > 4 wk (> 28 d) (W = 575.500, P = 0.003) with the median IgM levels (AU/mL) being highest in the survival group (Supplementary Figure 7).

DISCUSSION

The COVID-19 RT-PCR test is the most commonly used molecular test for the diagnosis of COVID-19 infection and is considered the gold standard test[12]. COVID-19 serology has emerged as one of the alternatives for diagnosing the COVID-19 disease. One of the meta-analyses by Chen *et al*[13] showed that the panel of IgG+ or IgM+ had a sensitivity of almost 79%, followed by IgG+ IgM+/- (73%), IgG+/- IgM+ (68%). Pooled specificities of these tests ranged from 98% to 100%. In our study also, in patients who had clinical COVID-19, almost 50% of patients were seropositive (IgM+ or IgG+).

Various studies have revealed that certain biochemical markers like IL-6 can be used as a prognostic marker for COVID-19[14]. The role of COVID serology in this aspect is less investigated upon. One of the retrospective studies done by Yan *et al*[15] showed that patients who had severe COVID-19 disease had higher COVID-19 IgG antibodies after 1 year. In this study also patients who were RT-PCR positive had statistically significant COVID-19 antibody serology. Also, Seropositivity for IgG increases as disease severity increases as shown in this study.

In one of the cross-sectional studies done in Iran, the study suggested that the patients who were IgG and IgM-positive had more severe symptoms compared to patients who had negative serology[16]. If we see the relationship between COVID-19 serology and complications, not many studies had been done in the past. This study had shown that patients who had higher COVID-19 IgG levels at three weeks had more severe ARDS and oxygen requirements compared to other patients. We also observed that there was a statistically significant difference in IgG antibody titers between the presence or absence of septic shock at three weeks. A similar trend was seen for ICU admissions and the need for mechanical ventilation. Also, in patients, who developed AKI there was more IgG seropositivity than IgM.

Previous studies by Liu *et al*[18], 2020, Zhang *et al*[19] showed that higher antibody (IgM and IgG) levels are seen in patients with severe and critical patients compared to mild-moderate patients[17-19]. Chen *et al*[20], 2021 study shows similar results as the above studies. However, the study showed antibody titer levels may vary and higher antibody titers were present in some mild-moderate category patients than in severe and critical patients. These findings are due to variations in serology to symptom onset interval[11,20,21]. The study also did not find a statistically significant correlation between antibody tires with AKI, mechanical ventilation, ICU requirement, septic shock, and mortality.

This study shows that higher body titers are associated with poor outcomes at a particular time serology to symptom onset interval. There are some limitations in this study first, it is a retrospective study, most of the patients in the study were not vaccinated and dynamic observation variation in antibody tires with the outcomes studied in a single patient. Second, there are limited patients in severe and critical patients compared to mild and moderate which may lead to biases in the results.

CONCLUSION

Serology (IgM and IgG) levels are high in RT-PCR positive group compared to clinical COVID-19. However, serology cannot be useful for the prediction of disease outcomes. The study also highlights the importance of doing serology at a particular time as antibody titers vary with the duration of the disease. In week intervals there was a significant correlation between clinical outcomes and serology on week 3.

ARTICLE HIGHLIGHTS

Research background

Predicting the severity of inflammatory response helps in managing critical patients using serology tests immunoglobulin G (IgG) and immunoglobulin M (IgM).

Research motivation

The importance of doing coronavirus disease (COVID) serology at a particular time as antibody titers may vary with the duration of the disease.

Research objectives

The objectivity was the correlation of the serology (IgM and IgG) with reverse transcriptase polymerase chain reaction (RT-PCR) status, disease severity (mild to critical), intensive care unit (ICU) admission, septic shock, acute kidney injury, and in-hospital mortality.

Research methods

This was a longitudinal study to correlate serum SARS-CoV-2 IgM and IgG serology with clinical outcomes in COVID-19 patients. We analyzed patient data from March to December 2020 for those who were admitted at All India Institute of Medical Sciences Rishikesh. Clinical and laboratory data of these patients were collected from the e-hospital portal and analyzed. A correlation was seen with clinical outcomes and was assessed using SPSS software.

Research results

Out of 494 patients, the mean age of patients was 48.95 ± 16.40 years and there were more male patients in the study (66.0%). The patients were classified as mild-moderate 328 (67.1%), severe 131 (26.8%), and critical 30 (6.1%). The mean duration from symptom onset to serology testing was 19.87 ± 30.53 d. In-hospital mortality was observed in 25.1% of patients. The seropositivity rate (*i.e.*, either IgG or IgM > 10 AU) was 50%. IgM levels (AU/mL) (W = 33428.000, $P \leq 0.001$) and IgG levels (AU/mL) (W = 39256.500, $P \leq 0.001$), with the median IgM/IgG levels (AU/mL), were highest in the RT-PCR-Positive group compared to RT-PCR-Negative clinical COVID-19. There was no significant difference between the two groups in terms of all other clinical outcomes (disease severity, septic shock, ICU admission, mechanical ventilation, and mortality).

Research conclusions

The study showed that serology levels are high in RT-PCR positive group compared to clinical COVID-19. The study also highlights the importance of doing serology at a particular time as antibody titers vary with the duration of the disease.

Research perspectives

The serology cannot be useful for the prediction of disease outcomes. In week intervals there is a significant correlation between clinical outcomes and serology on week 3.

FOOTNOTES

Author contributions: Panda PK, and Vivekanandhan V contributed to conceptualization; Panda PK contributed to methodology; Raina R contributed to software; Panda PK, Jain V, Suresh M, Omar BJ, and Kumar P contributed to validation; Raina R contributed to formal analysis; Saha S contributed to investigation; Panda PK contributed to resources; Suresh M contributed to data curation; Suresh M and Kumar P contributed to writing-original draft preparation; Panda PK and Omar BJ contributed to writing-review and editing; Panda PK contributed to visualization; Panda PK, Saha S, and Vivekanandhan V contributed to supervision; Panda PK, and Vivekanandhan V contributed to project administration; All authors have read and agreed to the published version of the manuscript.

Institutional review board statement: The Approval for this study was obtained from the institute ethics committee of All India Institute of Medical Sciences Rishikesh with approval no CTRI/2020/08/027169.

Informed consent statement: Consent is waived considering de-identification of the patient data.

Conflict-of-interest statement: We declare that we have no conflicts of interest and it's not funded.

Data sharing statement: It will be made available to others as required upon requesting the corresponding author.

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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S-Editor: Liu JH
L-Editor: A
P-Editor: Liu JH

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ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2023 May 27; 14(3): 62-71



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WJBC

World Journal of Biological Chemistry

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Bimonthly Volume 14 Number 3 May 27, 2023

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World Journal of Biological Chemistry

Bimonthly Volume 14 Number 3 May 27, 2023

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INDEXING/ABSTRACTING

The *WJBC* is now abstracted and indexed in PubMed, PubMed Central, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Yi-Xuan Cai*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Yun-Xiaojiao Wu*.

NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Vsevolod Gurevich, Jean-Marie Exbrayat, Chunpeng Craig Wan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1949-8454/editorialboard.htm

PUBLICATION DATE
May 27, 2023

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World J Biol Chem 2023 May 27; 14(3): 62-71

DOI: 10.4331/wjbc.v14.i3.62

ISSN 1949-8454 (online)

ORIGINAL ARTICLE

Basic Study

Comparison of the conventional tube and erythrocyte-magnetized technology in titration of red blood cell alloantibodies

Xue-Hua He, Hong Yan, Chun-Yan Wang, Xue-Yun Duan, Jia-Jia Qiao, Xiao-Jun Guo, Hong-Bin Zhao, Dong Ren, Jian-She Li, Qiang Zhang

Specialty type: Biochemistry and molecular biology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Mesa F, Spain; Yerke LM, United States

Received: March 14, 2023
Peer-review started: March 14, 2023
First decision: April 7, 2023
Revised: April 16, 2023
Accepted: May 15, 2023
Article in press: May 15, 2023
Published online: May 27, 2023

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Abstract

BACKGROUND

Erythrocyte alloantibodies are mainly produced after immune stimulation, such as blood transfusion, pregnancy, and transplantation, and are the leading causes of severe hemolytic transfusion reactions and difficulty in blood grouping and matching. Therefore, antibody screening is critical to prevent and improve red cell alloantibodies. Routine tube assay is the primary detection method of antibody screening. Recently, erythrocyte-magnetized technology (EMT) has been increasingly used in clinical practice. This study intends to probe the application and efficacy of the conventional tube and EMT in red blood cell alloantibody titration to provide a reference for clinical blood transfusion.

AIM

To investigate the application value of conventional tube and EMT in red blood cell alloantibody titration and enhance the safety of blood transfusion practice.

METHODS
A total of 1298 blood samples were harvested from blood donors at the Department of Blood Transfusion of our hospital from March 2021 to December 2022. A 5 mL blood sample was collected in tubing, which was then cut, and the whole blood was put into a test tube for centrifugation to separate the serum. Different red blood cell blood group antibody titers were simultaneously detected using the tube polybrene test, tube antiglobulin test (AGT), and EMT screening irregular antibody methods to determine the best test method.

RESULTS
Simultaneous detection was performed through the tube polybrene test, tube AGT and EMT screening irregular antibodies. It was discovered that the EMT screening irregular antibody method could detect all immunoglobulin G (IgG) and immunoglobulin M (IgM) irregular antibodies, and the results of manual tube AGT were satisfactory, but the operation time was lengthy, and the equipment had a large footprint. The EMT screening irregular antibody assay was also conducted to determine its activity against type O Rh (D) red blood cells, and the outcomes were satisfactory. Furthermore, compared to the conventional tube method, the EMT screening irregular antibody method was more cost-effective and had significantly higher detection efficiency.

CONCLUSION
With a higher detection rate, the EMT screening irregular antibody method can detect both IgG and IgM irregular antibodies faster and more effectively than the conventional tube method.

Key Words: Erythrocyte-magnetized technology; Conventional tube; Red blood cell alloantibodies; Transfusion reactions; Application

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Core Tip: Irregular antibody screening has long been a routine blood test for blood donors in numerous developed countries. However, only a few blood stations in China have tried using a saline medium for this type of screening. Monoclonal anti-A (B) is a standard reagent for ABO blood grouping, but false positive or false negative reactions can occur, reducing the accuracy of the test. With the improvement of diagnostic techniques and medical levels, the erythrocyte-magnetized technology (EMT) screening irregular antibody method has been gradually applied in a range of clinical settings. This study analyzed blood samples from voluntary blood donors to explore the application value and effect of the conventional tube method and EMT in red blood cell alloantibody titration, with the goal of providing valuable references to improve the safety of clinical blood transfusion.

Citation: He XH, Yan H, Wang CY, Duan XY, Qiao JJ, Guo XJ, Zhao HB, Ren D, Li JS, Zhang Q. Comparison of the conventional tube and erythrocyte-magnetized technology in titration of red blood cell alloantibodies. *World J Biol Chem* 2023; 14(3): 62-71
URL: https://www.wjgnet.com/1949-8454/full/v14/i3/62.htm
DOI: https://dx.doi.org/10.4331/wjbc.v14.i3.62

INTRODUCTION

Antibody screening aims to detect the presence of antibodies other than the ABO blood group in patients' serum or plasma by serum determination. This includes autoantibodies, drug antibodies, and allospecific antibodies to red blood cell blood groups[1,2]. Red blood cell alloantibodies, which are blood group antibodies other than anti-A and anti-B, are referred to as irregular antibodies. These antibodies are mainly produced after immune stimulation, such as blood transfusion, pregnancy, and transplantation, and are the primary cause of severe hemolytic transfusion reactions, difficulties in blood grouping, and complex matching[3-5]. Therefore, improving the screening rate of red blood cell alloantibodies is crucial, as it has significant implications for the safety of clinical blood transfusion.

Although many developed countries have been including irregular antibody screening in routine blood testing for blood donors, only a few blood stations in China have experimented with irregular antibody screening using saline media[6]. False positive or negative reactions sometimes occur using monoclonal anti-A (B) as a standard ABO blood grouping reagent[7]. The Collies automatic blood grouping system has recently been utilized in clinical practice employing erythrocyte-magnetized

technology (EMT), an international advanced technique. This technique magnetizes red blood cells, causing rapid sedimentation of red blood cells under the attraction of a magnetic field, thus replacing the serological centrifuge[8]. Irregular antibody screening using this instrument has been reported to be an indirect antiglobulin test (AGT) method that combines solid-phase packaging antiglobulin with EMT. The plasma to be tested was incubated with ready-to-use irregular antibody screening red blood cells, which can be sensitized by irregular antibodies in the plasma. In the presence of a magnetic field, magnetized red blood cells migrate to the bottom of the microplate and react with antiglobulin, forming an evenly distributed red blood cell layer[9,10]. Earlier studies have demonstrated that EMT can effectively mitigate the impact of centrifugation on experimental results, making the results more reliable and saving the costs associated with centrifuge calibration and maintenance[11,12].

Based on this, this study analyzed blood samples from voluntary blood donors to explore the application value and effect of the conventional tube and EMT in red blood cell alloantibody titration, hoping to provide valuable references for the safety of clinical blood transfusion.

MATERIALS AND METHODS

General information

We randomly selected 1298 blood donors from the Department of Blood Transfusion at our hospital between March 2021 and December 2022. The donors were between 18 and 55, with 612 males and 686 females. Among the donors, 897 (69%) were between 25 and 40. We separated serum from 5 mL of whole blood using centrifugation.

Materials and reagents

Reagents and instruments: Anti-human globulin cards (Jiangsu Libo Pharmaceutical Biotechnology Co., Ltd., batch number: 202208001), red blood cell antibody screening cell kits (batch number: 20187012, 20187014), and red blood cell antibody identification spectrum cells (REAGENS, batch number: 726000, 733000) were provided by Shanghai Blood Biomedicine Co., Ltd. Special centrifuge for blood immunology 2005-2 Zhuhai Bezo Biotechnology Co., Ltd. Polybrene came from reagents and instruments company of Zhuhai Bezo Biotechnology Co., Ltd. Collies 3 (QWALYS3) automatic blood analyzer (DIAGAST, France), Deakin (EvO-2) immunoassay sample adding system (TECAN, Switzerland). Shanghai Medical Equipment Co., Ltd. Digital explicit water bath, Heidolph oscillator (Titramax101) made in Germany, and HeShi centrifuge with hanging micro reaction plates in the German obstetrics department.

Eleven known irregular antibodies with known specificity against D, E, C, c, e, S, Fy', JK', M, N, and phosphatidylinositol antibody were prepared by the Institute of Blood Transfusion Technology, Taiyuan, China.

The TSFT puncture kit is mainly composed of low ionic medium, polybrene application solution, neutralization solution, and positive control (1gG anti-D), which are provided by Shanghai Blood Bio-Pharmaceutical Co., Ltd. and Zhuhai Bezo Biotechnology Co., Ltd., respectively.

Methods

The following procedures were used to screen and identify antibodies in blood donors using the all-tube method: Equal amounts of sera from 10 blood donors were mixed and identified for cell reactions with antibodies using the tube method Treponema pallidum particle test for antibody screening. If a positive reaction was observed, antibody screening was repeated on the 10 original samples before mixing using the traditional AGT. For those who tested positive, irregular antibodies were identified using antibody identification cells, and an absorption and diffusion test was performed if necessary to determine antibody specificity.

The tube polybrene test was conducted as follows[13]: One drop (50 μL) of reagent red blood cells was mixed with two drops (100 μL) of serum or plasma and incubated at room temperature for 15 s at 3000 rpm. The results were double-checked and recorded. Agglutination indicated a positive reaction, while the absence of agglutination indicated a negative reaction.

The tube AGT method was performed as follows[14]: After observing the results using the above methods, the red blood cell serum or plasma mixture was incubated at 37 °C for 30 min. The red blood cells were then washed three times with saline and finally, the packed red blood cells were suspended with two drops (100 mL) of antiglobulin and centrifuged. The outcomes were evaluated according to the same criteria as the upper water tube polybrene test method.

EMT screening for irregular antibodies[15]: With the use of the Collies automatic blood grouping system, with a special program matched with the original reagent, the instrument loads a 96-well ScreenLys microplate coated with antiglobulin, adds SONL isolation solution, diluent, 151 L magnetized screening cells, and 15 μL specimen plasma, completes the closed incubation at 37 °C after sample addition according to the set program, and completes the irregular antibody screening determination of

the specimen by magnetization shaking, photography, and interpretation. Figure 1 shows the test results.

Antibody potency assay: The irregular antibody was diluted by doubling with antibody diluent and reacted with the reagent red blood cells with corresponding positive antigens, and its titer was measured. The titer endpoint is the highest dilution with agglutination ≥ 1 +. These reagent red blood cells were also reacted with normal donor sera as a negative control test.

Statistical analysis

All data from this study were processed and analyzed using IBM SPSS 21.0 software (SPSS Inc., Chicago, IL, United States), and a t-test was harnessed to compare groups. If $P < 0.05$, statistical significance was confirmed.

RESULTS

Screening and identification results of irregular antibodies in blood donors

A total of 1298 random blood donors were screened using a commercial kit, and the polybrene test performed irregular antibody detection. The results revealed that 18 cases were positive, with a positive rate of 1.39%. Further identification showed the presence of Rh, MN, and other blood group system antibodies, as detailed in Table 1.

Comparison of sensitivity for detecting irregular antibodies with known specificity

A total of 11 known irregular antibody titers were detected in parallel by tube polybrene test, tube AGT, and EMT screening irregular antibody methods, respectively, while negative quality control tests were performed. It was found that all immunoglobulin G (IgG) and immunoglobulin M (IgM) irregular antibodies could be detected by the EMT screening irregular antibody method, and the manual tube AGT results in the control group were satisfactory, but the operation time was long, and the equipment occupied a large space (such as tubes and racks), as shown in Table 2.

Comparison of irregular antibody screening positive samples from the blood donor

The 18 positive samples selected from the blood donor spectrum were detected in parallel by the tube polybrene test, tube ACT, and EMT screening irregular antibody method. It was discovered that the reaction pattern of all sera and commercial screening cells or spectrum cells was consistent.

Effect of hemolysis and lipemia on irregular antibodies for EMT screening

The physical examination of blood donors discovered macroscopically significant severe hemolysis and lipemia samples. In equal proportion, the supernatant sera from these samples were mixed with low concentrations (titer 1:8) of IgG anti-D and AB sera (negative samples). In addition, their activities against type O Rh (D) red blood cells were determined by EMT screening irregular antibodies. The results indicated that the staining failed, but the cell membrane permeability remained normal, suggesting that the cells were active.

Comparison of cost of reagents, equipment, and manpower consumption

The consumption cost of screening irregular antibodies in the aforementioned blood donors was analyzed using the tube polybrene test, tube ACT and EMT screening methods. The cost was calculated by dividing the cost of each method by the number of blood donors tested. The outcomes showed that the EMT screening irregular antibody method cost less than 0.5 RMB per blood donor, while the tube polybrene test and tube AGT methods cost more than 5 RMB and 5.5 RMB per blood donor, respectively. These findings demonstrated that the EMT screening method was cost-effective and had advanced detection technology. Additionally, it allowed for sample concentration, resulting in improved work efficiency (see Table 3 for details).

DISCUSSION

At present, blood transfusion therapy, as a clinical treatment, is still irreplaceable in the treatment of some diseases. Although some advanced treatment options have significantly reduced the dependence on blood products in the treatment after being applied in clinical practice, hemolytic transfusion reactions and cross-matching difficulties caused by blood group antibodies other than ABO, that is, irregular antibodies, also occur from time to time[16,17]. Blood group antibodies destroy mismatched red blood cells in transfused blood or shorten their lifespan, producing hemolytic transfusion reactions, which may affect treatment outcomes or endanger the patient's life. Studies have unveiled that neonatal

Table 1 Specific distribution of irregular antibodies in 18 blood donors

Antibody specificity	Number of cases	Percentage of irregular antibodies (%)	IgG classification	Potency
Anti-D	7	38.91	IgG	2-64
Anti-E	2	11.11	IgG	16-32
Anti-cE	1	5.56	IgG	8
Anti-C	1	5.56	IgG	16
Anti-PI	4	22.22	IgM or IgG + IgM	8-16
Anti-M	1	5.56	IgG	4-32
Anti-N	1	5.56	IgG	8.32
Anti-Lea	1	5.56	IgG	4-16

Anti-D: Anti-D antibody; Anti-E: Anti-E antibody; Anti-cE: Anti-cE compound antigens antibody; Anti-C: Anti-C antibody; Anti-PI: Anti phosphatidylinositol antibody; Anti-M: Anti M antibody; Anti-N: Anti N antibody; Anti-Lea: Anti-Lea antibody; IgG: Immunoglobulin G; IgM: Immunoglobulin M.

Table 2 Results of irregular antibody titers detected in parallel by three detection methods

Test method	Specificity of irregular antibodies											
		D	E	C	c	e	S	Fy^b	Jk^a	M	N	P_1
Tube polybrene test	Potency	512	256	128	64	32	16	32	64	64	64	32
	Score	57	53	46	40	33	30	34	40	38	37	35
Tube AGT method	Potency	256	64	64	32	16	16	8	32	16	16	32
	Score	52	59	40	32	28	29	20	30	28	28	32
EMT screening irregular antibody method	Potency	512	256	128	64	16	16	32	64	64	64	32
	Score	56	54	64	41	31	29	33	40	38	36	34

D, E, C, c, e, S, Fy^b, Jk^a, M, N, P_1 is all IgG antibodies; Fy^b and Jk^a represent IgG type anti Fyb antibodies and IgG type anti Jk^a antibodies. EMT: Erythrocyte-magnetized technology; AGT: Antiglobulin test.

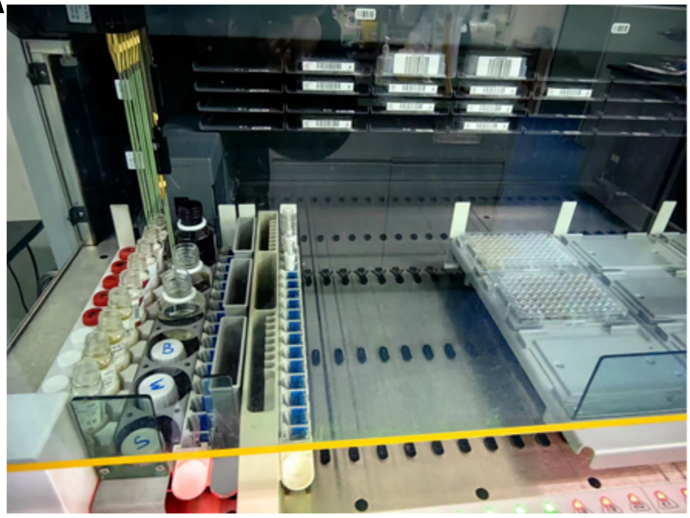
Table 3 Cost comparison of different methods

Methods	Cost (CNY/Donor)
EMT screening irregular antibody method	< 0.5
Tube polybrene test	> 5
Tube AGT method	> 5.5

EMT: Erythrocyte-magnetized technology; AGT: Antiglobulin test.

hemolytic disease caused by prenatal irregular blood group antibodies in pregnant women, especially Rh-HDN, has severe symptoms and often leads to serious harm[18,19]. Furthermore, monoclonal hyperactivity-A (B) reagent gas reportedly reacts with most red blood cells with high sensitivity and specificity[20]. Malformed hemolytic transfusion reactions caused by ABO blood group incompatibility are typically uncommon. In contrast, adverse transfusion reactions, neonatal hemolysis, and difficulty in blood grouping caused by irregular antibodies to ABO blood group accidents of red blood cells occasionally occur[21,22]. In addition, it has been internationally reported that monoclonal reagents can lead to false agglutination or false negative phenomenon during ABO blood group detection, and there have been corresponding reports in China in recent years[23]. Therefore, blood grouping alone is insufficient before transfusion, and antibody screening is necessary to ensure blood transfusion safety.

A

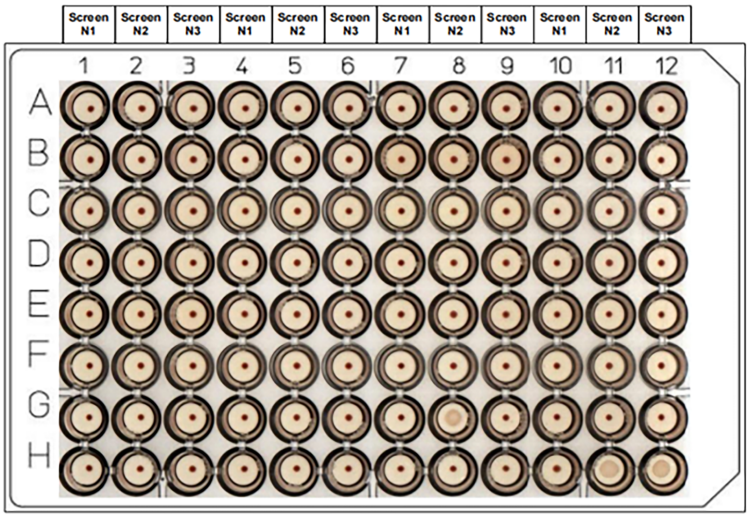


B

DIAGAST

Microplate number 6843032449601056 Irregular antibody screeıg

	Screen N1 1	Screen N2 2	Screen N3 3	Screen N1 4	Screen N2 5	Screen N3 6	Screen N1 7	Screen N2 8	Screen N3 9	creen N1 10	Screen N2 11	Screen N3 12
A	2110117840 - 0	2110117840 - 0	2110117840 - 0	2110115483 - 0	2110115483 - 0	2110115483 - 0	2110200310 - 0	2110200310 - 0	211020031021102077 87 - 0	- 0	2110207787 - 0	2110207787 - 0
B	2110118091 - 0	2110118091 - 0	2110118091 - 0	2110116979 - 0	2110116979 - 0	2110116979 - 0	2110207612 - 0	2110207612 - 0	211020761221102087 43 - 0	- 0	2110208743 - 0	2110208743 - 0
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DOI: 10.4331/wjbc.v14.i3.62 **Copyright** ©The Author(s) 2023.

Figure 1 Application of red blood cell magnetization technology in irregular antibody screening. A: Automatic blood group analyzer QWALYS 3 is used for irregular antibody screening; B: Erythrocyte blood group antibody identification cell reaction pattern table.

In recent years, with the development of medical technology, automatic blood grouping has been more and more widely used in the blood transfusion departments of major hospitals and blood collection institutions at all levels. It also plays a vital role in blood grouping tests, irregular antibody screening tests, and clinical cross-matching tests[24]. EMT is a new technology based on red blood cell magnetization, which has been maturely applied in immunodiagnosis, cell separation, protein purification, and nucleic acid extraction, and has also improved the automation rate of laboratories[25, 26]. Based on this, this study aimed to investigate the effect of the conventional tube and EMT in red blood cell alloantibody titration, hoping to provide some help for clinically safe blood transfusion.

This study collected 5 mL of whole blood from 1298 blood donors. The serum was separated by centrifugation. Different red blood cell blood group antibody titers were detected in parallel using the tube polybrene test, tube AGT, and EMT screening irregular antibody. Usually, voluntary blood donor samples are centrally collected. In order to save labor and reagents, 5-10 samples can be mixed for primary screening, and positive samples can be reexamined one by one to detect antibody-positive samples. However, because a few irregular antibodies with low titer in the mixed plasma still have the possibility of missed detection, the mixed plasma primary screening method is not suitable for the pretransfusion test of patients. In addition, it was found that all irregular antibodies of IgG and IgM nature were detected by EMT screening irregular antibody method. Nevertheless, the tube polybrene test method and tube AGT method did not detect irregular antibodies of low concentration, which may be related to the lack of sensitivity of IgM active blood group antibodies in low concentrations and at room temperature in the polybrene method and AGT.

Red cell alloantibodies are classified based on their properties as macromolecular IgM or small molecular IgG[27]. There are many methods to detect red blood cell antibodies, but standard tube methods can only detect IgM antibodies in most cases[28,29]. The results showed that the EMT screening irregular antibody method had clear advantages in determining the activity of IgG O Rh (D) red blood cell antibodies. In recent years, the use of traditional *in vitro* AGT has become more popular in China[30]. The findings in this paper indicate that the EMT screening irregular antibody method, the *in vitro* polybrene test method, and the *in vitro* AGT method can be utilied for routine screening of irregular antibodies in blood donors. Among these methods, the EMT screening irregular antibody method is more rapid, convenient, efficient, and less expensive.

In summary, compared with the *in vitro* polybrene test method and *in vitro* AGT method, the Collies automatic blood group system based on EMT for irregular antibody screening has the characteristics of more robust antibody specificity, more convenient operation, more accurate results, and complete lake source, which is suitable for large-scale screening of irregular antibodies in blood stations and is worthy of being widely popularized in clinical practice. Of course, more sensitive test techniques help to improve the antibody detection rate, but there may still be some antibodies that are not easy. In daily testing, the recorded history of checking past antibodies should be listed as a part of routine compatibility tests. Hospitals at all levels should actively understand the patient's blood transfusion history, pregnancy history, and current history and perform blood group and irregular antibody detection in advance so that the matching blood components can be selected for patients in time to ensure the safety and effectiveness of clinical blood transfusion.

CONCLUSION

Compared with the conventional tube method, the EMT-based Collies automatic blood group system for irregular antibody screening can more accurately screen its irregular antibody and has more substantial antibody specificity. In addition, it is more convenient to operate, with more accurate results, less cost consumed, and other characteristics, and is worthy of broader promotion in clinical practice.

ARTICLE HIGHLIGHTS

Research background

Magnetic red cell immunoseparation is a biomedical technique for separating and detecting small numbers of targeted cells. It has the advantages of high sensitivity, high precision, and easy operation and has been widely applied in the field of *in vitro* diagnostics and therapy.

Research motivation

Conventional separation methods can be time-consuming and involve complicated procedures, while magnetic red cell immunoseparation has the advantages of ease of use, high sensitivity, and precision. Therefore, this technology has been widely applied in *in vitro* diagnostics and therapy, attracting much attention from researchers and medical professionals.

Research objectives

This study aims to explore the application value of conventional test tubes and erythrocyte-magnetized technology (EMT) in red blood cell alloantibody titration to improve the safety of clinical blood transfusion.

Research methods

Parallel detection of antibody titers for different red blood cell blood groups using *in vitro* polyene test, tube antiglobulin test (AGT) and EMT screening for irregular antibodies.

Research results

The irregular antibody method for EMT screening could detect all immunoglobulin G and immunoglobulin M irregular antibodies, and the operation time is shorter than manual tube AGT. Furthermore, the EMT screening irregular antibody test was performed to detect its activity on O-type R (D) red blood cells, and the results reflected that it was normal. In addition, compared to the conventional tube method, the EMT screening method for irregular antibodies had lower costs and significantly higher detection efficiency.

Research conclusions

Compared with traditional *in vitro* methods, EMT screening for irregular antibodies has lower costs and significantly higher detection efficiency.

Research perspectives

The broad application prospects of red blood cell magnetization technology in medicine are evident. Technological advancements will further expand the application scope of this technology. For example, red blood cell magnetization technology can diagnose diseases like early cancer and cardiovascular diseases. In addition, this technology can be used for *in vivo* and *in vitro* research on the movement, interaction, and molecular processes of cells and pathogenic microorganisms.

FOOTNOTES

Author contributions: He XH and Yan H contributed equally; He XH, Qiao JJ, and Guo XJ designed the study; Zhao HB, and Ren D wrote the manuscript; Yan H, and Li JS reviewed and edited; Wang CY projected administration; Duan XY performed the experiments; Zhang Q analysed the data; All authors have read and agreed to the published version of the manuscript.

Supported by Project of Shanxi Provincial Health Commission, No. 2021144.

Institutional review board statement: The study was approved by Ethics Committee of the Third Hospital of Shanxi Medical University, No. 2018-003.

Institutional animal care and use committee statement: This study was not involved any animal experiments.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

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Country/Territory of origin: China

ORCID number: Qiang Zhang 0009-0008-7138-9099.

S-Editor: Fan JR
L-Editor: A
P-Editor: Cai YX

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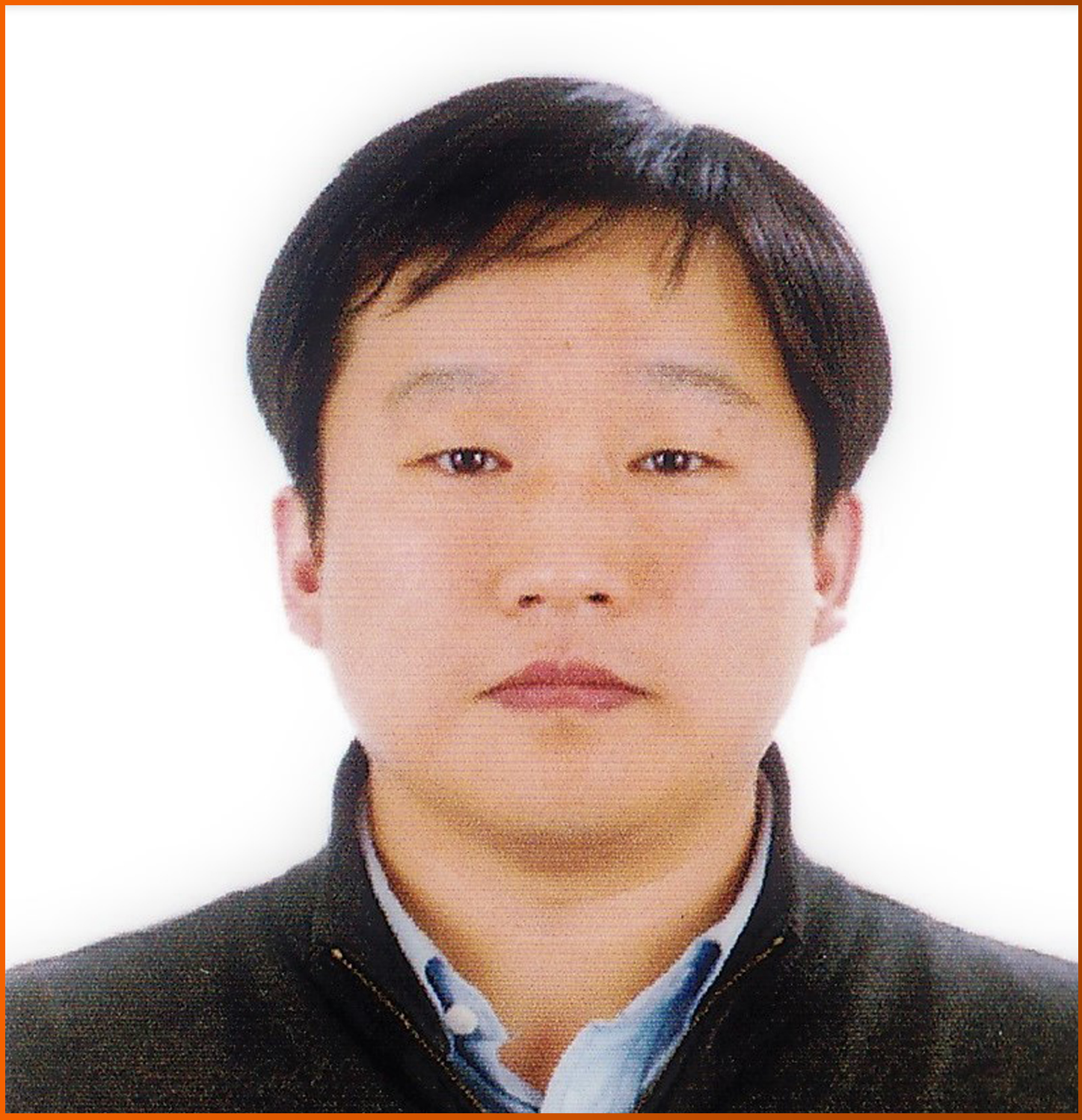


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ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2023 July 27; 14(4): 72-83



Published by Baishideng Publishing Group Inc

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World Journal of Biological Chemistry

Bimonthly Volume 14 Number 4 July 27, 2023

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INDEXING/ABSTRACTING

The *WJBC* is now abstracted and indexed in PubMed, PubMed Central, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Yi-Xuan Cai*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Yun-Xiaojiao Wu*.

NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Vsevolod Gurevich, Jean-Marie Exbrayat, Chunpeng Craig Wan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1949-8454/editorialboard.htm

PUBLICATION DATE
July 27, 2023

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World J Biol Chem 2023 July 27; 14(4): 72-83

DOI: 10.4331/wjbc.v14.i4.72

ISSN 1949-8454 (online)

ORIGINAL ARTICLE

Basic Study

In silico evidence of Remdesivir action in blood coagulation cascade modulation in COVID-19 treatment

Luis Gustavo Pagliarin, Lucca Miketen de Oliveira, Valentina Nunes Fontoura dos Anjos, Cristiano de Bem Torquato de Souza, Gabrielle Caroline Peiter, Cinthia Façanha Wendel, Anderson Dillmann Groto, Fabrício Freire de Melo, Kádima Nayara Teixeira

Specialty type: Chemistry, medicinal

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Jian X, China; Meng FZ, China

Received: December 30, 2022
Peer-review started: December 30, 2022
First decision: April 13, 2023
Revised: April 30, 2023
Accepted: July 3, 2023
Article in press: July 3, 2023
Published online: July 27, 2023

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Abstract

BACKGROUND

Coronavirus disease 2019 (COVID-19) has demonstrated several clinical manifestations which include not only respiratory system issues but also liver, kidney, and other organ injuries. One of these abnormalities is coagulopathies, including thrombosis and disseminated intravascular coagulation. Because of this, the administration of low molecular weight heparin is required for patients that need to be hospitalized. In addition, Remdesivir is an antiviral that was used against Middle East Acute Respiratory Syndrome, Ebola, Acute Respiratory Syndrome, and other diseases, showing satisfactory results on recovery. Besides, there is evidence suggesting that this medication can provide a better prognosis for patients with COVID-19.

AIM

To investigate *in silico* the interaction between Remdesivir and clotting factors, pursuing a possibility of using it as medicine.

METHODS

In this *in silico* study, the 3D structures of angiotensin-converting enzyme 2 (ACE2), Factor I (fibrinogen), Factor II (prothrombin), Factor III (thromboplastin),

Factor V (proaccelerin), Factor VII (proconvertin), Factor VIII (antihemophilic factor A), Factor IX (antihemophilic factor B), Factor X (Stuart-Prower factor), and Factor XI (precursor of thromboplastin (these structures are technically called receptors) were selected from the Protein Data Bank. The structures of the antivirals Remdesivir and Osetalmivir (these structures are called ligands) were selected from the PubChem database, while the structure of Atazanavir was selected from the ZINC database. The software AutoDock Tools (ADT) was used to prepare the receptors for molecular docking. Ions, peptides, water molecules, and other ones were removed from each ligand, and then, hydrogen atoms were added to the structures. The grid box was delimited and calculated using the same software ADT. A physiological environment with pH 7.4 is needed to make the ligands interact with the receptors, and still the software Marvin sketch® (ChemAxon®) was used to forecast the protonation state. To perform molecular docking, ADT and Vina software was connected. Using PyMol® software and Discovery studio® software from BIOVIA, it was possible to analyze the amino acid residues from receptors that were involved in the interactions with the ligands. Ligand tortions, atoms that participated in the interactions, and the type, strength, and duration of the interactions were also analyzed using those software.

RESULTS

Molecular docking analysis showed that Remdesivir and ACE2 had an affinity energy of -8.8 kcal/moL, forming a complex with eight hydrogen bonds involving seven atoms of Remdesivir and five amino acid residues of ACE2. Remdesivir and prothrombin had an interaction with six hydrogen bonds involving atoms of the drug and five amino acid residues of the clotting factor. Similar to that, Remdesivir and thromboplastin presented interactions *via* seven hydrogen bonds involving five atoms of the drug and four residues of the clotting factor. While Remdesivir and Factor V established a complex with seven hydrogen bonds between six antiviral atoms and six amino acid residues from the factor, and Factor VII connected with the drug by four hydrogen bonds, which involved three atoms of the drug and three residues of amino acids of the factor. The complex between Remdesivir and Factor IX formed an interaction *via* 11 hydrophilic bonds with seven atoms of the drug and seven residues of the clotting factor, plus one electrostatic bond and three hydrophobic interactions. Factor X and Remdesivir had an affinity energy of -9.6 kcal/moL, and the complex presented 10 hydrogen bonds and 14 different hydrophobic interactions which involved nine atoms of the drug and 16 amino acid residues of the clotting factor. The interaction between Remdesivir and Factor XI formed five hydrogen bonds involving five amino acid residues of the clotting factor and five of the antiviral atoms.

CONCLUSION

Because of the *in silico* significant affinity, Remdesivir possibly could act in the severe acute respiratory syndrome coronavirus 2 infection blockade by interacting with ACE2 and concomitantly act in the modulation of the coagulation cascade preventing the hypercoagulable state.

Key Words: Clotting factors; Coagulating blood cascade; COVID-19 treatment; Remdesivir; SARS-CoV-2

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Core Tip: In the initial period of the emergence of coronavirus disease 2019, bioinformatics tools filled the need for rapid knowledge about the severe acute respiratory syndrome coronavirus 2 and research in search of drug treatment, since experimental research takes time that was not available. Bioinformatics continues to be used for the same purpose in this study, remembering that this methodology does not rule out either *in vivo* or *in vitro* testing.

Citation: Pagliarin LG, de Oliveira LM, dos Anjos VNF, de Souza CBT, Peiter GC, Façanha Wendel C, Dillmann Groto A, Freire de Melo F, Teixeira KN. *In silico* evidence of Remdesivir action in blood coagulation cascade modulation in COVID-19 treatment. *World J Biol Chem* 2023; 14(4): 72-83
URL: https://www.wjgnet.com/1949-8454/full/v14/i4/72.htm
DOI: https://dx.doi.org/10.4331/wjbc.v14.i4.72

INTRODUCTION

At the end of 2019, the health system of Wuhan, China reported an alarming growth in the number of patients with severe acute respiratory syndrome (SARS) triggered by a new coronavirus, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[1-3]. A highly contagious disease, coronavirus disease 2019 (COVID-19), spread across several countries[4] in the following months, culminating in the decree of a pandemic by the World Health Organization on March 11, 2020[1]. Due to the high transmissibility of the disease and the increase in the number of deaths, several governments imposed measures of using face masks, isolation of new cases and social distancing, and restriction of circulation, with the closure of schools and universities, prohibition of agglomerations, as well as decreeing the

paralyzing of non-essential activities in an attempt to slow down the spread of the disease[2].

The most widespread form of contagion of SARS-CoV-2 is through the contact with droplets of oropharynx secretions from an infected person. In addition, the transmission of the virus is aggravated by its incubation time, which varies from 3 to 14 d, and by the probability of asymptomatic people or those with mild symptoms transmitting the disease[5]. COVID-19 may not manifest symptoms (asymptomatic patients), manifest mild, moderate, or severe symptoms, and may progress to severe pneumonia and death[6,7]. In addition to the respiratory system, other organs and systems may also present injury from viral infection, such as the liver, kidneys, heart, and neural system[8]. Thus, although respiratory failure is the main factor leading to death, causes linked to organ failure, thrombosis, and disseminated intravascular coagulation (DIC), accompanied by intense inflammatory response, are also responsible for a great decline in the health status of patients[9]. Of the COVID-19 positive patients who had DIC, only 0.6% survived, which demonstrates the high lethality at this stage of the disease[10,11].

The immune response to SARS-CoV-2 infection is associated with the prevalence of coagulation pathway activation and the large increase in the production of pro-inflammatory cytokines, tumor necrosis factor (TNF)-alpha and IFN-gamma, a condition called "cytokine storm". In addition to acting in the conversion reaction of fibrinogen into fibrin to form the clot, thrombin can also act in increasing inflammation, since these inflammatory events are related to high concentrations of this enzyme and low concentrations of proteinase activated receptors, antithrombin III, and protein C [12]. Although the coagulopathies observed in these patients are reminiscent of DIC and other thrombotic events, they have characteristics inherent to COVID-19[9]. D-dimer, a product of fibrin degradation, when elevated, has been associated with a higher mortality rate[13]. Some studies have reported that the increase in D-dimer and fibrinogen in the bloodstream is associated with a poor prognosis in patients with COVID-19[14,15]. At other times, these parameters are not consistent with the severity of pathological conditions[9,10,16]. In any case, the severity of COVID-19 promotes the imbalance of coagulation and anticoagulation factors, in addition to precipitating the formation of thrombosis, DIC, respiratory deterioration, and multiple organ failure[11].

The International Society of Thrombosis and Hemostasis recommends that low molecular weight heparin (LMWH) be administered in patients in need of hospitalization, that prophylactic doses of LMWH should be administered in all hospitalized patients, and that D-dimer, prothrombin, activated partial thromboplastin, and fibrinogen levels be monitored daily[17]. The adoption of LMWH was recommended since the tests in patients treated with prophylactic doses had better prognoses and a reduction of mortality when compared to the untreated ones[18].

Near the end of 2020, studies were underway for the development of prophylactic vaccines against COVID-19. Currently, several vaccines are available to the world population, with different vaccination strategies in relation to the number of doses. As for the present time, the epidemic is controlled and vaccine prophylaxis contributed to this scenario. However, the situation of the COVID-19 pandemic is still unpredictable. There is no effective vaccine in fact, given reinfections, or specific antiviral drugs to treat patients with severe COVID-19[19].

The treatment of severe cases, accompanied by DIC, represents a challenge in the therapeutic process of safeguarding the lives of patients. Although there are studies on experimental drugs considered promising, the lack of proven and effective treatment for severe cases of COVID-19 raises the possibility of repurposing drugs, *i.e.*, the reuse of existing drugs, in order to optimize time in controlling disease progression and reduce the cost spent by health systems[20,21].

Remdesivir (GS-5734) is a broad-spectrum antiviral that has been shown to be effective in containing the replication of pathogens that cause Middle East Acute Respiratory Syndrome, Acute Respiratory Syndrome (SARS-CoV), Ebola, and other infectious agents[22-24]. This drug is a prodrug of adenosine and its metabolic product, the active triphosphate GS-443902, is analogous to ATP and inhibits by competition the enzyme RNA-dependent RNA polymerase[25-28]. Studies conducted with COVID-19-positive patients, who were treated with Remdesivir, showed satisfactory results of faster recovery when compared to patients not treated or those who received placebo[29-32]. In addition, a report validated results of hospitalized patients with severe COVID-19 who were treated with Remdesivir, suggesting that the drug can prevent the progression of the severity of infection, since in the treated group there was less evolution of cases[30].

The analysis of data and hypotheses about the action and effectiveness of drugs, such as Remdesivir, can be aided by bioinformatics tools that can act as a method of understanding and predicting the molecular interactions of the drug with possible molecules that may be involved in the process of viral infection or in the development of symptoms and pathological conditions. In this context, some *in silico* studies have proposed candidate drugs against some target structures of SARS-CoV-2 that have been deposited in the databases until then[33,34]. Based on the hypothesis that Remdesivir acts in symptomatic events of COVID-19 and according to the premises that patients' coagulopathies are of great severity and that Remdesivir is an approved and useful drug in the treatment of patients affected by COVID-19, this study aimed to verify by molecular docking the interaction of Remdesivir with coagulation cascade factors, seeking to contribute to the best understanding of its action and application of COVID-19 pharmacological therapy.

MATERIALS AND METHODS

Preparation of receptors and ligands

The three-dimensional structures of the human proteins were obtained from the Protein Data Bank (PDB) (rcsb.org/pdb): ACE2 (PDB:6VW1), Factor I (fibrinogen; PDB:3GHG), Factor II (prothrombin; PDB:3U69), Factor III (thromboplastin; PDB:5W06), Factor V (proaccelerin; PDB:1CZT), Factor VII (proconvertin; PDB:1KLJ), Factor VIII (antihemophilic factor A; PDB:2R7E), Factor IX (antihemophilic factor B; PDB:6MV4), Factor X clotting factor (Stuart-Prower factor; PDB:4Y71), and Factor XI (thromboplastin precursor; PDB:5QTU). These structures, technically called receptors, were prepared for molecular docking using the AutoDock Tools 4 (ADT) software (autodock.scripps.edu). Any ions, peptides, and

molecules previously attached to the receptor, as well as water molecules derived from the crystallization technique for resolution of the structure by X-ray diffraction, were removed at this stage. Subsequently, missing hydrogen atoms were added to the receptors, and the location and dimensions of the grid box were determined. The grid box features x, y, and z coordinates were built with grid spacing of 1.0 Å using the AutoGrid component of the ADT software. Grid box data and coordinates were used in molecular fitting.

The three-dimensional structures of the ligands (drugs) were obtained from two different sources. In the PubChem database (pubchem.ncbi.nlm.nih.gov), the three-dimensional structures of the antivirals Remdesivir (PubChem 121304016) and Oseltamivir (PubChem 65028) were obtained. Oseltamivir was administered with good results in patients with COVID-19 without hypoxia[35].

In the ZINC database (zinc12.docking.org), the three-dimensional structure of the antiviral Atazanavir (ZINC 3941496) was obtained. The ligands interact with their receptors in a physiological environment with pH 7.4. Thus, to reliably simulate their interaction, a calculation was performed to predict the protonation state using Marvin sketch® software (ChemAxon®). Protonated ligands were prepared for molecular docking using ADT software which detects the torsion points of the inhibitors and calculates their torsion angle.

Molecular docking

Molecular docking simulations were performed using ADT software connected to AutoDock Vina v.1.2.0. software[36]. This software associates two components - a search algorithm and a scoring function. The algorithm is responsible for searching for possible combinations in the bindings, and exploring the degrees of rotational, translational, and conformational freedom of the ligand as well as proteins. Then the scoring function is used to choose the best binding modes. These functions are obtained according to the strength fields of molecular mechanics and empirical parameters from free energy calculations, so an affinity energy is calculated. An affinity energy < -6.0 kcal/moL is considered the cutoff point for stable interactions[37]. The results are based on the first conformer of the ligands, with the reference root-mean-square deviation of atomic position equaling zero[38]. The analyses of amino acid residues of the receptors involved in the interactions with the ligands, as well as the ligand tortions, atoms involved in the interactions, and the type, strength, and duration of the interactions, were performed using the PyMol® (Schrödinger, Inc.) and BIOVIA Discovery studio® (Dassault Systemes) software.

RESULTS

Among the complexes formed between protein and antiviral drugs, the most stable ones were selected, according to the affinity energy value. Simulations between Atazanavir, Remdesivir, and Osetalmivir with ACE2, Factors I (fibrinogen) and II (protrombin) indicated significantly greater stability between the three proteins and Remdesivir (Table 1). Due to the most promising data of Remdesivir, the analyses followed with this drug. Molecular docking was performed between Remdesivir and other clotting factors and the drug interacted with all tested factors *in silico* (Table 2). Remdesivir and ACE2 formed a complex through eight hydrogen bonds involving seven atoms of Remdesivir and five amino acid residues of ACE2 (Table 3); affinity energy was -8.8 kcal/moL. In molecular docking simulations, Remdesivir was used in its protonation state at pH 7.4, in which the amount of the drug is 99.85% protonated.

The association between Remdesivir and prothrombin occurred through six hydrogen bonds involving atoms of the drug and five amino acid residues of the clotting factor (Table 4); similarly, Remdesivir/thromboplastin complex was stabilized by seven hydrogen bonds involving five atoms of the drug and four residues of the clotting factor.

Factor V and Remdesivir formed a complex through seven hydrogen bonds between six antiviral atoms and six amino acid residues from the factor. Four hydrogen bonds were observed between Remdesivir and Factor VII. These bonds involved three atoms of the drug and three residues of amino acids of the protein (Table 4). The Remdesivir/Factor IX complex was stabilized by 11 hydrophilic bonds (hydrogen bonds) involving seven atoms of the drug and seven residues of the clotting factor, one electrostatic bond, and three hydrophobic interactions.

Factor X and Remdesivir were associated with an energy of affinity equal to -9.6 kcal/moL. The complex was established and maintained by hydrogen bonds (10) and hydrophobic interactions (14) of varied types involving nine atoms of the drug and 16 amino acid residues of the clotting factor (Table 5). Remdesivir and Factor XI formed a complex by the interaction between five amino acid residues of the clotting factor and five antiviral atoms by means of five hydrogen bonds. Complexes with significant affinity energy, whose physiological behavior is relevant to explain the supposed action of Remdesivir as a treatment of symptoms resulting from COVID-19, are shown in Figure 1.

DISCUSSION

Since the affinity energy value between two structures must be less than −6.0 kcal/moL for the interaction to be considered stable[37], it is possible to point out that Remdesivir was the only antiviral that presented itself within this premise. Remdesivir is a pro-drug, analogous to adenosine, which acts by inhibiting the viral replication of several viral families, including the Coronaviridae family. With regard to its mechanism of action, it is observed that it competes with the endogenous ATP for incorporation at viral RNA through RNA-dependent RNA polymerase, triggering the termination of the chain[39].

Table 1 Energy of affinity of ligand/receptor complexes obtained in molecular docking and characteristics of interactions between Remdesivir and angiotensin-converting enzyme 2

Receptor/ligand complex	Energy of affinity (kcal/moL)
ACE2/Remdesivir	-8.8
ACE2/Osetalmivir	-5.7
ACE2/Atazanavir	-3.9
Fibrinogen/Remdesivir	-8.5
Fibrinogen/Osetalmivir	-6.3
Fibrinogen/Atazanavir	-4
Prothrombin/Remdesivir	-8.4
Prothrombin/Osetalmivir	-6
Prothrombin/Atazanavir	-4.1

ACE2: Angiotensin-converting enzyme 2.

Table 2 Characteristics of interactions between Remdesivir and angiotensin-converting enzyme 2

	Interaction[1]	Distance (Å)	Bond type
ACE2/Remdesivir	N-18:GLU-398	3.3	Hydrogen bond
	O-29:HIS-378	2.5	Hydrogen bond
	O-30:ALA-348	2.4	Hydrogen bond
	O-30:ALA-348	3.3	Hydrogen bond
	O-34:ALA-348	3.2	Hydrogen bond
	O-36:ASP-350	2.3	Hydrogen bond
	O-37:ASP-350	3.4	Hydrogen bond
	O-40:ARG-393	3.5	Hydrogen bond

[1]Remdesivir: Residue. ACE2: Angiotensin-converting enzyme 2; O: Oxygen; N: Nitrogen; GLU: Glutamate; ALA: Alanine; ASP: Aspartate; ARG: Arginine.

Table 3 Energy of affinity of ligand/receptor complexes obtained *in silico*

Ligand/receptor complex	Energy of affinity (kcal/moL)
Remdesivir/tissue factor (thromboplastin)	-8.2
Remdesivir/Factor V (proacelerin)	-6.8
Remdesivir/Factor VII (proconvertin)	-7.0
Remdesivir/Factor IX (anti-hemophilic B)	-6.4
Remdesivir/Factor X	-9.6
Remdesivir/Factor XI	-8.3

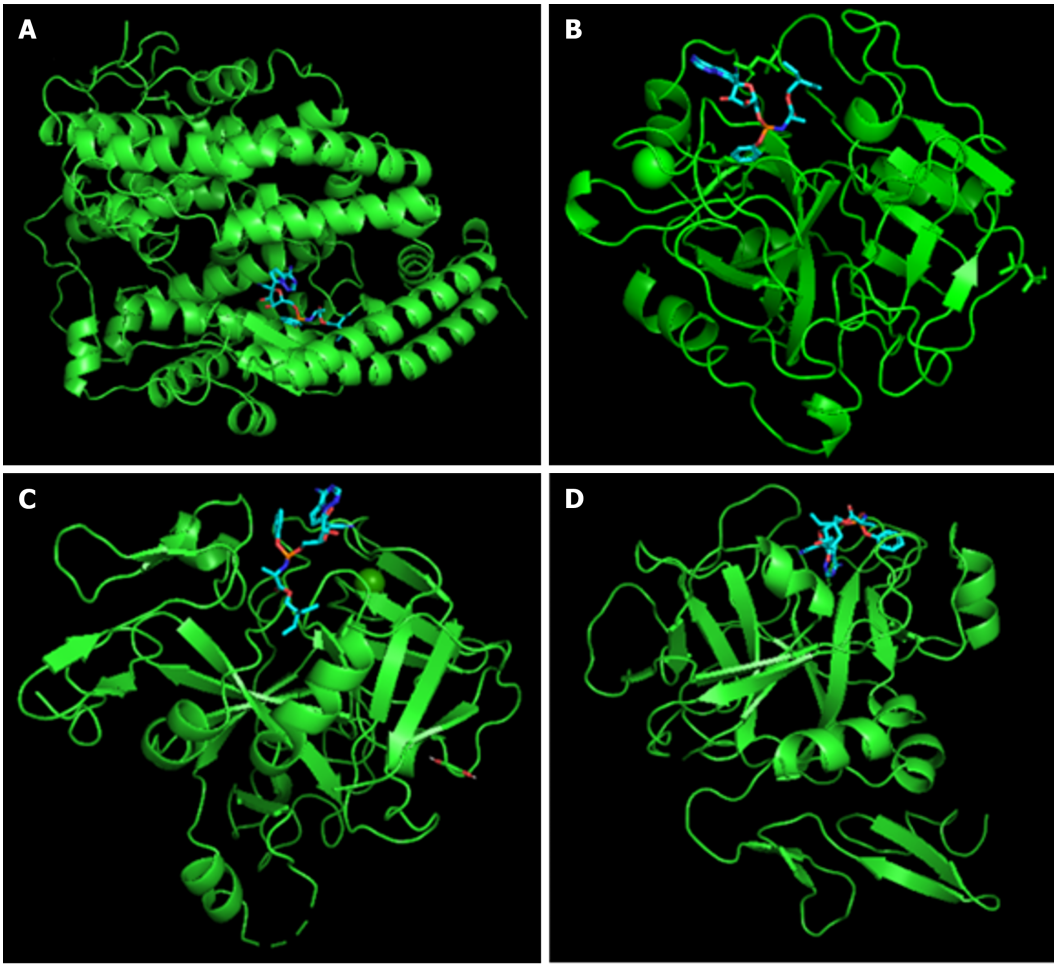
The *in silico* analyses indicated that Remdesivir interacts with clotting factors, so in the investigation of the consequences of its use in patients with COVID-19, it is important to analyze the possible routes of action of the drug, as well as the related effects, since during infection by SARS-CoV-2, a broad spectrum of changes from bleeding to thrombus formation can occur[40]. Therefore, for the understanding of the interference of Remdesivir in the coagulation, it is essential to understand its physiological functioning.

It is important to highlight that the pathways generated by the cadence action of the different agents that are part of the hemostatic process are not fixed to a single series, occurring simultaneously and in ways not completely understood *in vivo*[41]. As described in an article by a researcher of University Hospital of Wales (Cardiff, United Kingdom), hemostasis is triggered by endothelial damage, with collagen exposure and tissue factor, and this process is accompanied by

Table 4 Binding characteristics of Remdesivir/Factor II and Remdesivir/Factor VII complexes

Complex	Interaction[1]	Distance (Å)	Bond type
Remdesivir/Factor II II (protrombin)	O-23: GLY-548	2	Hydrogen bond
	O-3: GLY-548	3.2	Hydrogen bond
	N-15: ASN-463	2.6	Hydrogen bond
	O-35: SER-525	2.4	Hydrogen bond
	O-35: HIS-363	2.6	Hydrogen bond
	O-3: GLY-550	2	Hydrogen bond
Remdesivir/Factor VII (Proconvertin)	O-3: ASP-60	3.5	Hydrogen bond
	O-3: THR-98	3.1	Hydrogen bond
	O-2: THR-98	3.6	Hydrogen bond
	O-2: GLY-97	3.2	Hydrogen bond
	N-17: GLY-216	3.1	Hydrogen bond

[1]Remdesivir: Residue. O: Oxygen; N: Nitrogen; ASP: Aspartate; ASN: Asparagine; THR: Threonine; SER: Serine; HIS: Histidine; GLY: Glycine.



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Figure 1 Remdesivir/receptor complexes. A: Remdesivir/angiotensin-converting enzyme 2; B: Remdesivir/Factor II; C: Remdesivir/Factor VII; D: Remdesivir/Factor X. Receptors are shown in green and Remdesivir is shown in blue/red.

endothelial secretion that results in localized vasoconstriction reflex, reducing local blood flow[42].

Table 5 Binding characteristics of Remdesivir/Factor X complex

Interaction[1]	Distance (Å)	Bond type
N-18: ALA-190	3.33	Hydrogen bond
N-18: GLY-219	3.44	Hydrogen bond
N-23: GLY-193	2.3	Hydrogen bond
N-23: GLN-192	3.35	Hydrogen bond
O-29: SER-195	2.92	Hydrogen bond
O-29: SER-195	2.29	Hydrogen bond
O-29: SER-214	2.78	Hydrogen bond
O-29: HIS-57	2.92	Hydrogen bond
O-29: HIS-57	2.53	Hydrogen bond
O-30: HIS-57	2.93	Hydrogen bond
Aromatic ring C-3: TRP-215	4.21	Pi-stacking
Aromatic ring C-3: TRP-215	4.03	Pi-stacking
Aromatic ring C-3: TYR-99	5.06	Pi-stacking
Aromatic ring C-3: PHE-174	5.13	Pi-stacking
Aromatic ring C-8: CYS-191 and GLN-192	4.57	Amide pi-stacking
Aromatic ring C-31: CYS-191 and GLN-192	4.31	Amide pi-stacking
Aromatic ring C-8: TRP-215 and GLY-216	4.53	Amide pi-stacking
Aromatic ring C-31: TRP-215 and GLY-216	4.89	Amide pi-stacking
C-2: LYS-96	3.96	Alkyl
C-2: TYR-60	5.05	Pi-alkyl
C-2: PHE-94	5.39	Pi-alkyl
C-2: PHE-94	4.87	Pi-alkyl
Aromatic ring C-8: CYS-220	5.47	Pi-alkyl
Aromatic ring C-31: ALA-190	5.29	Pi-alkyl

[1]Remdesivir: Residue. O: Oxygen; N: Nitrogen; C: Carbon; ALA: Alanine; GLY: Glycine; SER: Serine; HIS: Histidine; TRP: Tryptophan; TYR: Tyrosine; PHE: Phenylalanine; CYS: Cysteine; LYS: Lysine.

Based on the results of molecular docking, it was possible to observe that in addition to the stable interaction with ACE2, Remdesivir also interacted, with significant affinity energy, with clotting factors of the intrinsic, extrinsic, and common pathways. It is emphasized that Factors V (common pathway) and IX (intrinsic pathway) had less significant interactions (> -7 kcal/moL) with the drug in relation to the other clotting factors, which may result in a lower interference in the cascade from the inhibition of these two factors and, consequently, in anticoagulation.

However, there was significant interaction (-7.0 kcal/moL) between Remdesivir and Factor VII, an initial factor of the cascade. Activated Factor VII (Factor VIIa) joins the tissue factor, forming a complex that activates Factor IX, leading to thrombin production; this same complex activates more Factor VII molecules, so even with a low initial level of this factor, its effectiveness is satisfactory due to the action of the factor/tissue factor complex[43]. In this respect, exclusively for the possible inhibition of Factor VII by Remdesivir, a significant alteration in the cascade does not necessarily occur, because the release of tissue factor is an essential condition for the formation of the complex and occurs in case of endothelial damage, increased during the pro-inflammatory state induced by SARS-CoV-2 infection[44]. In addition, low levels of Factor VIIa are sufficient to feed back the track.

Between Remdesivir and Factor X (common pathway), an interaction with an affinity energy value of -9.6 kcal/moL was recorded, which is the most significant interaction observed in this study, so this would be the most stable and most favorable interaction to occur *in vivo* and may be an important means of inhibiting the coagulation cascade. Secondary hemostasis is formed by the extrinsic and intrinsic pathways, activated by different factors, which converge in the activation of Factor X, resulting in a common pathway, and inhibition of this factor has been shown to prevent the progression of the cascade in baboon models[45]. In the common pathway, Factor Xa associated with Factor Va (activated by thrombin) cleaves prothrombin into thrombin. Thrombin (Factor IIa), which potentiates secondary hemostasis, along with Ca^{2+}, activates Factor XIII and also cleaves fibrinogen into fibrin, forming polymers. Associated with Factor XIIIa,

fibrin polymers form cross-links. In the convergence of the two pathways, the activation of Factor X allows the proteolytic cleavage of prothrombin into thrombin, which converts fibrinogen (soluble) into fibrin (insoluble); activates Factor XIII, which establishes cross-links in the fibrin network; potentiates the coagulation cascade by catalyzing Factors VIII and V; potentiates the coagulation cascade by catalyzing Factors VIII and V; and promotes platelet activation[46]. Thus, additional anticoagulatory effects could occur due to the relevance of Factor X in hemostasis and its interaction with Remdesivir.

Meanwhile, Factors I, II, III, and XI also connected to Remdesivir with significant affinity energies (between -9.0 and -8.0 kcal/moL), so these factors would be possible sites of action of the drug in the coagulation cascade. Factor XIa in the presence of Ca^{2+} and, separately, Factor VIIa activate factor IX, which, together with Factor VIIIa, activates Factor X[47]. Thus, combined with inhibition of Factor X, all these possible interactions with the drug would result in anticoagulation effects, which would be additional to the main mechanism of antiviral action of Remdesivir.

In view of the possibility of interaction of Remdesivir with components of hemostasis, it is necessary to discuss the possible pharmacodynamic interactions between this drug and anticoagulants that have been widely used for the treatment of patients with COVID-19, including heparins, warfarin, and new oral anticoagulants (NOAC).

About the class of heparins, represented by unfractionated heparin (UFH), LMWH, and Fondaparinux, it is contextualized that these compounds do not present intrinsic anticoagulant activity, but they bind to antithrombin to trigger a higher rate of inhibition of coagulation proteases[48]. Considering the three representatives, among the pharmacodynamic differences, the influence of variation on the number of chains of saccharides that make up the molecules stands out, in which the length of these molecules influences the binding and inactivation capacity of specific components of the coagulation cascade, conferring particularities to each molecule. For example, in the case of UFH, to potentiate thrombin inhibition (Factor IIa), chains composed of at least 18 units of saccharides (approximate molecular weight of 5400 Da) are required. By presenting in their composition molecules with an average weight of 15000 Da, virtually all UFH molecules are long enough for this function. On the other hand, LMWH, advantageously, has a greater capacity to potentiate the inhibition of Factor Xa than thrombin, and this results from its average molecular weight being between 4500 to 5000 Da, resulting in the preference of inhibition of Factor Xa in relation to thrombin. In relation to Fondaparinux, it accelerates only the inhibition of Factor Xa by antithrombin, and its length is insufficient to bind antithrombin to thrombin, which justifies the selectivity of action[49].

The NOAC, represented by the drugs Dabigatran, Rivaroxabana, Apixabana, and Edoxabana, target the same clotting factors, with the first being an active compound that binds competitively and reversibly to the active thrombin site (Factor IIa), preventing procoagulant activity, while the others acting as direct inhibitors of Factor Xa.

Considering these particularities, it can be inferred that Remdesivir has the potential for synergistic interaction with the aforementioned drugs. In this sense, due to the stable affinity energy (-9.6 kcal/moL) of Remdesivir/Factor Xa complex, UFH, LMWH, Fondaparinux, Rivaroxaban, Apixaban, and Edoxabana may have their effects pronounced when associated with the antiviral[50]. Similarly, this effect can occur with Dabigatran and heparins, since they are connected to Factor IIa, but with less emphasis on LMWH. This results from the occurrence of a stable binding complex from Remdesivir to thrombin (-8.4 kcal/moL), resulting in a possible therapeutic contribution.

Regarding warfarin, its mechanism of action involves the inhibition of coagulation cascade components, Factors II, VII, IX, and X, which are dependent on vitamin K for its activation. Inhibition is the result of blocking the enzyme vitamin K epoxide reductase, and warfarin prevents the conversion of oxidized vitamin K epoxide to its reduced form, vitamin K hydroquinone. As a consequence, there is inhibition of vitamin K-dependent carboxylation, since the hydroquinone form would act as a cofactor for gamma-glutamyl carboxylase, an enzyme responsible for the carboxylation process, dependent on vitamin K, of clotting factors[51,52]. After carboxylation, Factors II, VII, IX, and X are able to bind to Ca^{2+} and interact with the anionic surface of phospholipids. With conversion blockage, there is no link between the factors and calcium, resulting in the absence of interaction with platelets, preventing the continuity of the cascade.

Considering the mechanism of action of warfarin, possible synergistic drug interactions between warfarin and Remdesivir can be inferred. The possibility lies in the observation, *in silico*, of stable binding between Remdesivir and Factors II, VII, IX, and X, with energy of affinity values, respectively, of -8.4 kcal/moL, -7.0 kcal/moL, -6.5 kcal/moL, and -9.6 kcal/moL.

Similar to the *in silico* data, case reports were observed in the medical literature, despite the low level of evidence, and these studies strengthen the hypothesis of a drug interaction between Remdesivir and warfarin, due to the performance of both in the coagulation cascade. In the first observation of changes in the international normalized ratio (INR), a marker used for analysis of the extrinsic and common pathways, resulting from a possible interaction between the two drugs, a case report describes an INR that increased 2 d after the start of Remdesivir administration. The laboratory parameter remained elevated during remdesivir therapy and began to decrease after completion of treatment, reaching the therapeutic target of 2-3 after 5 d of the last dose of Remdesivir. In addition, the authors mentioned that the probability score calculated on the Naranjo scale was 6, suggesting that the increase in INR observed was secondary to a probable drug interaction between warfarin and Remdesivir[53].

In addition, Landayan and colleagues reported a potential unwell-understood interaction between warfarin and Remdesivir with dexamethasone. The application of the Drug Interaction Probability Scale to this case resulted in a score of 5, highlighting a potential drug interaction. According to the authors, this probable interaction is demonstrated by a marked increase of INR within 24 to 48 h after the beginning of the combination (Remdesivir plus dexamethasone) in two patients with a history of stable INR[54].

In another case report, Yao *et al*[55] described an increase in INR after the onset of Remdesivir administration to treat pneumonia due to COVID-19 in a patient on stable chronic warfarin therapy. The INR remained supratherapeutic after discontinuation of Remdesivir and returned to therapeutic levels approximately 8 d after discontinuation. The Naranjo Drug Adverse Reaction Probability Scale was 4, indicating a possible adverse reaction to the drug.

CONCLUSION

In silico analyses indicate that Remdesivir behaves as an antiviral by interacting with ACE2 with considerable affinity, and thus competing with the virus and hindering cellular infection. At the same time, this drug is able to bind with affinity and stability to several clotting factors, which would prevent the activation of the blood coagulation cascade. In cases of severe COVID-19 with the possibility of DIC, Remdesivir could act synergistically with anticoagulants to modulate hypercoagulation states and prevent further harm to patients.

ARTICLE HIGHLIGHTS

Research background

The need for a treatment for coronavirus disease 2019 (COVID-19) has prompted some commercial drugs to be tested off-label. The evaluation of off-label drugs has provided incomplete information about their efficacy and gaps about long-term and chronic adverse effects. Remdesivir is an example of an off-label drug that showed promising results in the treatment of COVID-19, but may have other benefits that have not been explored and could be studied *in silico*.

Research motivation

The motivation for this study arose from the need to provide data that could help inform studies on Remdesivir, since this drug has not been evaluated through standard clinical trials for COVID-19. Remdesivir has met clinical trials for the treatment of another viral disease, and when used for the treatment of a different disease, both benefits and side effects can arise. Since our team observed the affinity of this antiviral for clotting factors *in silico*, we hypothesized that it might have benefits not only for preventing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, but also for treating symptoms of severe COVID-19, such as disseminated intravascular coagulation.

Research objectives

The aim of the research was to evaluate whether Remdesivir has interaction affinity with clotting factors and to compare these interactions with the interaction with key receptors in SARS-CoV-2 infection to infer possible pharmacological activities.

Research methods

The study was performed in a computational environment using molecular docking and bioinformatics tools that allow to mimic the rational behavior of Remdesivir based on its chemical characteristics. The affinity energy and chemical bonds of Remdesivir with target molecules were analyzed and used to extrapolate *in silico* information to the human physiological environment.

Research results

The *in silico* results on the activity of Remdesivir in the coagulation cascade are promising when considering severe cases of COVID-19, but they need to be evaluated *in vivo*. *In silico* data show that Remdesivir can bind to various clotting factors with different affinities; this suggests that this drug can prevent coagulation by acting at several distinct points of the cascade, which increases its effectiveness when disseminated intravascular coagulation is triggered by viral infection.

Research conclusions

Our research team suggests that Remdesivir may be an off-label drug used as an antiviral for the treatment of COVID-19, but it can also be used to treat severe symptoms of the disease; however, this needs to be confirmed by *in vivo* testing.

Research perspectives

The research perspective is to evaluate, *in silico*, other off-label drugs suggested for the treatment of COVID-19.

FOOTNOTES

Author contributions: Pagliarin LG, Oliveira LM, Anjos VNF, and Souza CBT performed the experiments, analyzed the results, and wrote the manuscript; Peiter GC, Wendel CF, and Groto AD performed the experiments; Melo FF performed the critical analysis of the results; Teixeira KN interpreted the data, performed the critical analysis of the results, corrected the manuscript, and coordinated the study; all authors approved the final version of the manuscript.

Institutional review board statement: This study used data from three-dimensional structures deposited in public online databases.

Conflict-of-interest statement: The authors state that there is no conflict of interest of any kind with regard to this study.

Data sharing statement: This study was developed *in silico* and no patient data or data from medical records were used in this study.

Open-Access: This article is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers.

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S-Editor: Liu JH
L-Editor: Wang TQ
P-Editor: Cai YX

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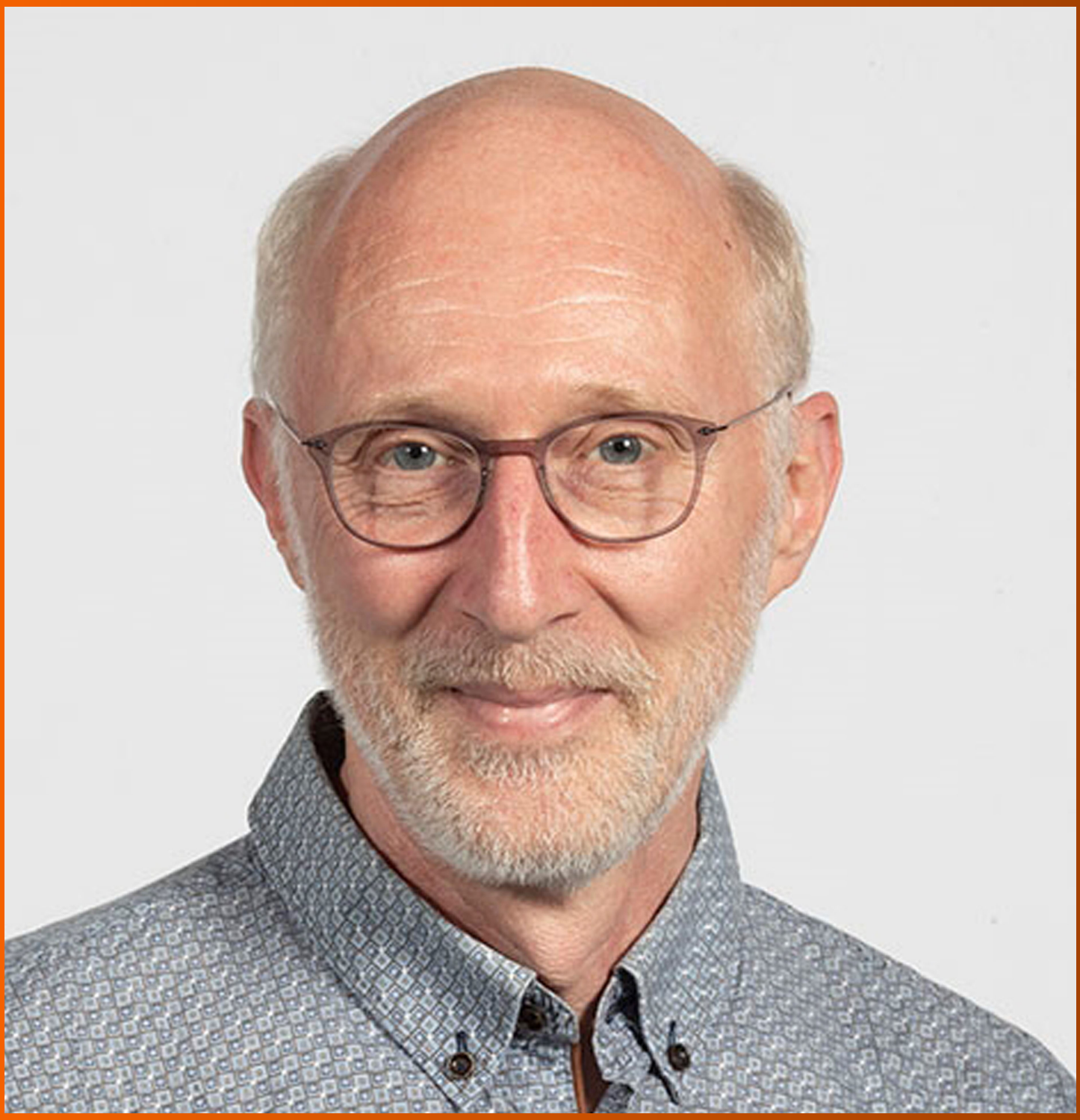


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ISSN 1949-8454 (online)

World Journal of *Biological Chemistry*

World J Biol Chem 2023 October 17; 14(5): 84-98



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Bimonthly Volume 14 Number 5 October 17, 2023

ORIGINAL ARTICLE

Basic Study

84 Protein arginine methyltransferase 6 is a novel substrate of protein arginine methyltransferase 1

Cao MT, Feng Y, Zheng YG

World Journal of Biological Chemistry

Bimonthly Volume 14 Number 5 October 17, 2023

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INDEXING/ABSTRACTING

The *WJBC* is now abstracted and indexed in PubMed, PubMed Central, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Yi-Xuan Cai*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Yun-Xiaojiao Wu*.

NAME OF JOURNAL
World Journal of Biological Chemistry

ISSN
ISSN 1949-8454 (online)

LAUNCH DATE
July 26, 2010

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Vsevolod Gurevich, Jean-Marie Exbrayat, Chunpeng Craig Wan

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1949-8454/editorialboard.htm

PUBLICATION DATE
October 17, 2023

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World J Biol Chem 2023 October 17; 14(5): 84-98

DOI: 10.4331/wjbc.v14.i5.84

ISSN 1949-8454 (online)

ORIGINAL ARTICLE

Basic Study

Protein arginine methyltransferase 6 is a novel substrate of protein arginine methyltransferase 1

Meng-Tong Cao, You Feng, Y George Zheng

Specialty type: Biochemistry and molecular biology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Arumugam VA, India; Chen Z, China

Received: July 19, 2023
Peer-review started: July 19, 2023
First decision: August 31, 2023
Revised: September 8, 2023
Accepted: September 26, 2023
Article in press: September 26, 2023
Published online: October 17, 2023

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Abstract

BACKGROUND

Post-translational modifications play key roles in various biological processes. Protein arginine methyltransferases (PRMTs) transfer the methyl group to specific arginine residues. Both PRMT1 and PRMT6 have emerges as crucial factors in the development and progression of multiple cancer types. We posit that PRMT1 and PRMT6 might interplay directly or in-directly in multiple ways accounting for shared disease phenotypes.

AIM

To investigate the mechanism of the interaction between PRMT1 and PRMT6.

METHODS

Gel electrophoresis autoradiography was performed to test the methyltranferase activity of PRMTs and characterize the kinetics parameters of PRMTs. Liquid chromatography-tandem mass spectrometryanalysis was performed to detect the PRMT6 methylation sites.

RESULTS

In this study we investigated the interaction between PRMT1 and PRMT6, and PRMT6 was shown to be a novel substrate of PRMT1. We identified specific arginine residues of PRMT6 that are methylated by PRMT1, with R106 being the major methylation site. Combined biochemical and cellular data showed that PRMT1 downregulates the enzymatic activity of PRMT6 in histone H3 methylation.

CONCLUSION

PRMT6 is methylated by PRMT1 and R106 is a major methylation site induced by PRMT1. PRMT1 methylation suppresses the activity of PRMT6.

Key Words: Posttranslational modification; Arginine methylation; Protein arginine

methyltransferase 1; Protein arginine methyltransferase 6; Cross-talk; Protein-protein interaction

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Core Tip: We reported the interplay between protein arginine methyltransferase (PRMT) 1 and PRMT6, and PRMT6 is a substrate of PRMT1. The major methylation site in PRMT6 is R106 for PRMT1 catalysis and the methylation by PRMT1 regulates the enzymatic activity of PRMT6. This study is important for understanding the cross-talking relationship between PRMTs.

Citation: Cao MT, Feng Y, Zheng YG. Protein arginine methyltransferase 6 is a novel substrate of protein arginine methyltransferase 1. *World J Biol Chem* 2023; 14(5): 84-98
URL: https://www.wjgnet.com/1949-8454/full/v14/i5/84.htm
DOI: https://dx.doi.org/10.4331/wjbc.v14.i5.84

INTRODUCTION

Protein arginine methylation is a widespread post-translational modification that regulates various biological processes in cellular physiology[1,2]. Protein arginine methyltransferases (PRMTs) act as the controller of arginine methylation that transfers the methyl group from S-adenosyl methionine (SAM) to specific arginine residues on histone or nonhistone protein substrates[3-6]. The PRMT members are categorized into three types according to the stereochemical specificity of their product[7,8]. The type I enzymes [PRMT1, PRMT2, PRMT3, coactivator associated arginine methyltransferase (CARM1), PRMT6, and PRMT8] catalyze the formation of ω-NG-monomethyl arginine (MMA) and ω-N^G, N^G-asymmetric dimethylarginine (ADMA)[9,10]. The type II enzymes (PRMT 5 and PRMT9) catalyze the formation of MMA and ω-N^G, N'^G-symmetric dimethylarginines[11,12]. PRMT7 belongs to the type III enzyme and exclusively generates MMA[13].

Upregulation of PRMTs expression level is commonly observed in clinical oncology and is correlated with poor prognosis of patients[14-17]. Therefore, PRMTs emerge as potential novel molecular targets in cancer therapy development[18-21]. PRMT1 and PRMT6 both belong to the Type I PRMT family and the activities of PRMT1 and PRMT6 have been associated with distinct cellular functions. PRMT1 contributes to most of the ADMA in proteins[22] and plays key roles in regulating such biological processes as transcriptional regulation, signal transduction, cell proliferation, DNA damage repair owing to its action on numerous histone and non-histone substrates. On the chromatin, PRMT1 generates ADMA marks on histone H4 arginine 3, H2AR3, and H2AR11[23-25]. Currently, seven PRMT1 isoforms (PRMT1-v1 to v7) have been identified: PRMT1-v1 and PRMT1-v7 are predominantly located in the nucleus, while PRMT1-v2 is primarily situated in the cytoplasm[26]. In gene expression regulation, PRMT1 is an activating transcription cofactor[27]. PRMT6 is another type-I PRMT member and is involved in a broad spectrum of biological and pathological processes, such as transcriptional regulation, DNA repair, cell movement, embryonic stem cell pluripotency, human immunodeficiency virus pathogenesis, and cardiovascular-associated diseases[28-33]. PRMT6 generates ADMA modifications on H2AR29, H3R2, H3R17 and H3R42[30,34-38]. When PRMT6 adds H3R2me2a to the promoters, it suppresses transcription; however, when H3R2me2a is added to enhancers, PRMT6 promotes transcription[39]. The subcellular localization analysis shows PRMT6 is predominantly present in the nucleus[40].

Both PRMT1 and PRMT6 have emerged as crucial factors in the development and progression of multiple cancer types such as breast cancer, colorectal cancer, lung cancer, and bladder cancer. Notably, experimental knockdown of PRMT1 and PRMT6 using siRNAs has been shown to significantly inhibit the growth of bladder and lung cancer cells[41]. The tumor-promoting roles of PRMT1 and PRMT6 in oncology are executed through their methylation of histone and non-histone substrates[41-45]. Expression profile analysis suggests that PRMT1 and PRMT6 are both involved in multiple pathways which are fundamental for cell proliferation[41]. We posit that PRMT1 and PRMT6 might interplay directly or in-directly in multiple ways accounting for shared disease phenotypes. Thereby, neutralizing these enzymes' activities or their interactions may interfere with tumor progression and lead to anticancer beneficial effects.

The structural analysis of PRMTs using X-ray crystallography has revealed a consistent homodimeric structure across all the type I PRMT isoforms, representing the active unit for their catalytic activity[46-48]. PRMT5 homodimer is further extended into a hetero-octameric state with methylosome protein 50 included in the complex[49]. In addition to homodimerization, several studies have provided evidence for the existence of PRMTs heterological complex formation with other PRMT members. For instance, Pak *et al*[50] found that PRMT1 forms heterodimers or oligomers with PRMT2, and this interaction synergistically enhances the catalytic activities of both enzymes. PRMT8 was shown to form homodimers and heterodimers with PRMT1[51]. CARM1 was found to interact with PRMT5 to repress human γ-globin gene expression[52]. PRMT5 was found to interact with PRMT6 to promote colorectal cancer progression[53]. However, the structures, mechanisms, and consequences of those interactions and their downstream effects on gene regulation and cellular processes require substantial investigation. In the present study, we reported a novel heteromeric interaction between PRMT1 and PRMT6. Remarkably, our new finding showed that PRMT6 acts as a new substrate of PRMT1, and the arginine methylation induced by PRMT1 led to a decrease in the enzymatic activity of PRMT6.

MATERIALS AND METHODS

Cell culture and transfection

About 0.3 × 10^6 human embryonic kidney 293T cells were seeded in the 6-well culture plate containing 2 mL of 1X Dulbecco's modified Eagle's medium (Cellgro) supplemented with 10% Fetal Bovine Serum (Cellgro) before incubating them at 37 ℃ with 5% CO_2. When about 80% confluence was reached, 1 × 10^6 cells were obtained. Transfection was performed using Lipofectamine 3000 (L3000008, Thermofisher) reagent. For the overexpression of PRMT1 or PRMT6, 2.5 μg HA-PRMT1 or Myc-PRMT6 or the mixture of HA-PRMT1 and Myc-PRMT6 was added in 125 μL of Opti-MEM medium with 5 μL P3000 reagent to get the diluted DNA. 10 μL of L3000008 reagent was diluted by 125 μL of Opti-MEM medium. Diluted DNA was mixed with diluted L3000008 at 1:1 ratio. After 15 min, the DNA-lipid complex was added to the cells. For the knock down of PRMT1, 5 μg shPRMT1 or shScramble were incubated with the reagents instead. The mixtures were added into each well of the 6-well plate to transfect HEK-293T cells.

Coimmunoprecipitation of PRMT1 and PRMT6

Myc-PRMT6 single-transfected HEK-293T cells or HA-PRMT1 and Myc-PRMT6 co-transfected 293T cells were collected by centrifugation at 1500 rpm at 4 ℃ for 10 min, washed with 1 mL [phosphate-buffered saline (PBS), Cellgro] twice, and lysed in M-PER mammalian protein extraction reagent (Thermo Scientific) containing 1% protease inhibitor cocktail (Thermo Scientific). The lysate was frozen and thawed once and centrifuged at 12000 rpm for 20 min. The supernatant was aliquoted with 100 μg protein and immunoprecipitated with (1): 2 μg anti-HA antibody or mouse [immunoglobulin G (IgG), Santa Cruz] at 4 ℃ for 2 h followed by 50 μL protein A/G plus agarose beads (Santa Cruz) at 4 ℃ overnight, and or (2): 2 μg anti-myc antibody (Origene) or mouse IgG at 4 ℃ for 2 h followed by 50 μL protein A/G plus agarose beads at 4 ℃ overnight. The beads were washed with 500 μL TBS buffer with 0.05% tween-20 for 3 times. The protein on the beads were eluted with sodium dodecyl sulfate (SDS) loading buffer and separated on 12 % SDS-polyacrylamide gel electrophoresis (PAGE). Then the protein bands were immunoblotted with: (1) Anti-PRMT6 (Santa Cruz); (2): anti-PRMT1; (3): anti-HA antibody, and or (4) anti-Myc antibody.

Methyltransferase activity of PRMTs

Radioactive methyltransferase assays were conducted to test the methylation of PRMT6 and its activity on histone H3. The reactions were carried out at 30 ℃ in a reaction buffer containing 50 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 8.0), 50 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and 0.5 mmol/L dithienothiophene (DTT) 3 μM PRMT1 or H3.1 (New England Biolabs) or H3.3 (New England Biolabs) together with 20 μM ^{14}C-SAM (Perkin Elmer) were incubated with 1 μM PRMT6 for 1 h. Control reaction with only PRMT6 and ^{14}C-SAM was performed to test its automethylation activity. The reactions were quenched with SDS loading buffer and the methylated products were separated by 12% SDS-PAGE. The radioactive gel was dried and stored for 16 h for a phosphor image scan. The methylated bands were quantified with ImageJ for data analysis.

For characterization of PRMT6 methylation mediated by PRMT1, the varied concentration of PRMT6 (0-4 μM) was incubated with 10 μM ^{14}C-SAM with 0.2 μM PRMT1^{WT} or PRMT1^{E153Q} at 30 ℃ for 1 h. For time-dependent PRMT6 methylation assay, 1.5 μM of PRMT6 and 10 μM of ^{14}C-SAM were incubated with 0.4 μM of PRMT1^{WT} or PRMT1^{E153Q}. The reactions were quenched at different time points (0-120 min) by SDS loading buffer. The methylation intensity data were normalized according to the reading of 5 μM ^{14}C-BSA in liquid scintillation and the PRMT6 automethylation intensity was subtracted from the total PRMT6 methylation intensity in the presence of PRMT1 to derive the data of methylation mediated by PRMT1. For comparison of PRMT6 mutant methylation, 5 μM of each PRMT6 mutant was incubated with 30 μM ^{14}C-SAM with or without 1 μM PRMT1 at 30 ℃ for 2 h. The methylation intensity data were normalized against PRMT6^{WT}.

Pull down assay of PRMT6

Amylose resin was washed with 5 volumes of column buffer to remove the storage buffer. 20 μg PRMT1 was incubated with the amylose beads under 4 ℃ for 30 min while rotating. The amylose resin with PRMT1 was centrifuged at 500 g for 5 min, the supernatant was discarded and the beads were washed by PBS 3 times. After that, 20 μg PRMT6 was incubated with the amylose beads bond with PRMT1 or amylose beads only under 4 ℃ for 4 h while rotating. The resin was centrifuged and washed by PBS 5 times to remove the excess PRMT6 that not binding with PRMT1. The pulled-down proteins were eluted by SDS-PAGE loading buffer and a western blot was performed to detect PRMT1 and PRMT6.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of the PRMT 6 methylation sites

The methylation sites on PRMT6, either mediated by PRMT1 or by itself, were detected by in-gel digestion and LC-MS/MS analysis following the methylation reaction. 5.5 μM PRMT6 and 200 μM regular SAM were incubated with 1 μM PRMT1 at 30 ℃ for 3 h in the same reaction buffer as mentioned above. SDS loading dye was added to quench the reaction and 10% SDS-PAGE was conducted to separate the protein. The PRMT6 band was cut from the gel, washed with distaining solution (800 μL), 0.1 M NH_4CO_3 in 50% acetonitrile (400 μL), and 100% acetonitrile (60 μL), and vacuum dried. Then the methylated PRMT6 sample was sent to UAB Proteomics and Mass Spectrometry Facility (Birmingham, AL 35294) for in-gel digestion (Trypsin) and LC-MS/MS detection.

Site-directed mutagenesis of PRMT6

The individually designed primers for the site-directed mutagenesis of potential methylation sites of PRMT6 were synthesized and desalted by integrated DNA technologies. The forward and reverse primers are *5'-gaagatggcgcggagaag-*

gaggcggccctggag-3′ and *5′-ctccagggccgcctccttctccgcgccatcttc-3′* for PRMT6-R29K, *5′-gaactgggcagcactgaaaggcaagacggtactgg-3′* and *5′-ccagtaccgtcttgcctttcagtgctgcccagttc-3′* for PRMT6-R82K, *5′-cccaggccggggccaagcgc-gtgtacgcgg-3′* and *5′ -ccgcgtacacgcgcttggc-cccggcctggg-3′* for PRMT6-R106K, *5′-ggagggctt-cgccacgaagtgtctcatgggccactc-3′* and *5′-gagtgg-cccatgagacacttcgtggcga-agccctcc-3′* for PRMT6-R228K, *5′-gctccgtcctccacgcgaaaaccaagtggctga-agg-3′* and *5′-ccttcagccacttggttttcgcgtggaggacg-gagc-3′* for PRMT6-R174K, *5′-cggtccacgaggagttgatcgcggaccgc-3′* and *5′-gcggtccgcgat-caactcctcgtggaccg-3′* for PRMT6-M60L, *5′-ctc-ctgcac-gagtccgcgctgagctccgtcctc-3′* and *5′-gaggacg-gagctcagcgcggactcgtgcaggag-3′* for PRMT6-M166A. Mutation of each codon was accomplished by polymerase chain reaction (PCR) amplification with native pfu polymerase, synthesized primers, and His6 × PRMT6-pET28a (+) as a template. Following PCR reactions, Dpn1 was added to digest the original methylated plasmid. Then the plasmid with each mutation was heat-shock transformed to XL1blue super-competent cells, which were selected on agar plates with Kanamycin at 37 ℃ overnight. The single colonies were picked up and incubated in 4 mL LB media with Kanamycin for 16 h. The mutant DNA was extracted with Wizard Plus SV Minipreps DNA purification system (Promega) and the correct mutations were confirmed by DNA sequencing with Genscript Inc.

Expression and purification of PRMT1, PRMT1 mutant, PRMT6 and PRMT6 mutants

His-tagged PRMT1, maltose-binding protein (MBP)-tagged PRMT1, His-tagged PRMT6, and the mutants were expressed by recombinant DNA technology in E. coli, and then affinity-purified with nickel-nitrilotriacetic acid (Ni-NTA) his binding resin (Novagen). PRMT1-pET28b or PRMT6/mutant-pET28a (+) plasmid was transformed into E. coli BL21 (DE3) (Stratagene) through heat shock. One colony of the bacteria was inoculated into 4 mL LB media containing kanamycin or ampicillin and incubated at 37 ℃ overnight. The next day, the culture was added to 1 L of LB media and incubated until OD_{595} reached 0.6-0.8. Protein expression was induced with 0.3 mmol/L isopropyl beta-D-1 thiogalactopyranoside at 16 ℃ overnight. After that, the culture was centrifuged at 5000 rpm for 10 min and the cell pellet was resuspended in lysis buffer [25 mmol/L Na-HEPES, pH 8.0, 150 mmol/L NaCl, 1 mmol/L $MgSO_4$, 5% ethylene glycol, 5% glycerol, and 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF)]. Cell lysis was carried out by high-pressure cell disruptor. The lysate was centrifuged and then the supernatant was loaded onto Ni-NTA beads equilibrated with column buffer (25 mmol/L Na-HEPES (pH 7.0), 300 mmol/L NaCl, 1 mmol/L PMSF, and 30 mmol/L imidazole). Following protein loading, the beads were washed thoroughly with 10 volumes of column buffer and 10 volumes of washing buffer (25 mmol/L Na-HEPES (pH 7.0), 300 mmol/L NaCl, 1 mmol/L PMSF, and 70 mmol/L imidazole), and then the bound protein was eluted with elution buffer (25 mmol/L Na-HEPES (pH 7.0), 300 mmol/L NaCl, 1 mmol/L PMSF, 100 mmol/L EDTA, and 200 mmol/L imidazole). Different eluants were checked on 12% SDS-PAGE gel. Elution fractions with target protein were combined and concentrated with 10000 molecular weight cut-off centrifugal filters (Millipore), and then dialyzed against storage buffer (25 mmol/L Na-HEPES (pH 7.0), 300 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L DTT and 10% glycerol) at 4 ℃. After dialysis, protein concentration was determined using Bradford assay. The final protein samples were aliquoted, flash-frozen, and stored at -80 ℃. For each enzymatic assay, a fresh aliquot was melted on ice and diluted to the assay concentration as needed.

Steady-state kinetics characterization of PRMT6 mutants

The steady-state kinetics of selected PRMT6 mutants were also studied in the radioactive assays at 30 ℃ in the same reaction buffer. The concentration is 0.6 μM for the PRMT6 mutant and 30 μM for ^{14}C-SAM. Synthesized H3 (1-31) peptide (sequence: *ARTKQTARKSTGGKAPRKQLATKVARKSAPA*) was used as a substrate, the concentration of which ranged from 0 to 200 μM. The reaction was initiated by adding the enzyme once the other components were incubated at 30 ℃ for 5 min. After continuous incubation for 60 min, the reaction was quenched by spreading the reaction mixture onto the surface of the P81 filter paper (Whatman). The paper was washed with 50 mmol/L $NaHCO_3$ (pH 9.0) and air dried. Then liquid scintillation was performed to quantify the amount of methylated products.

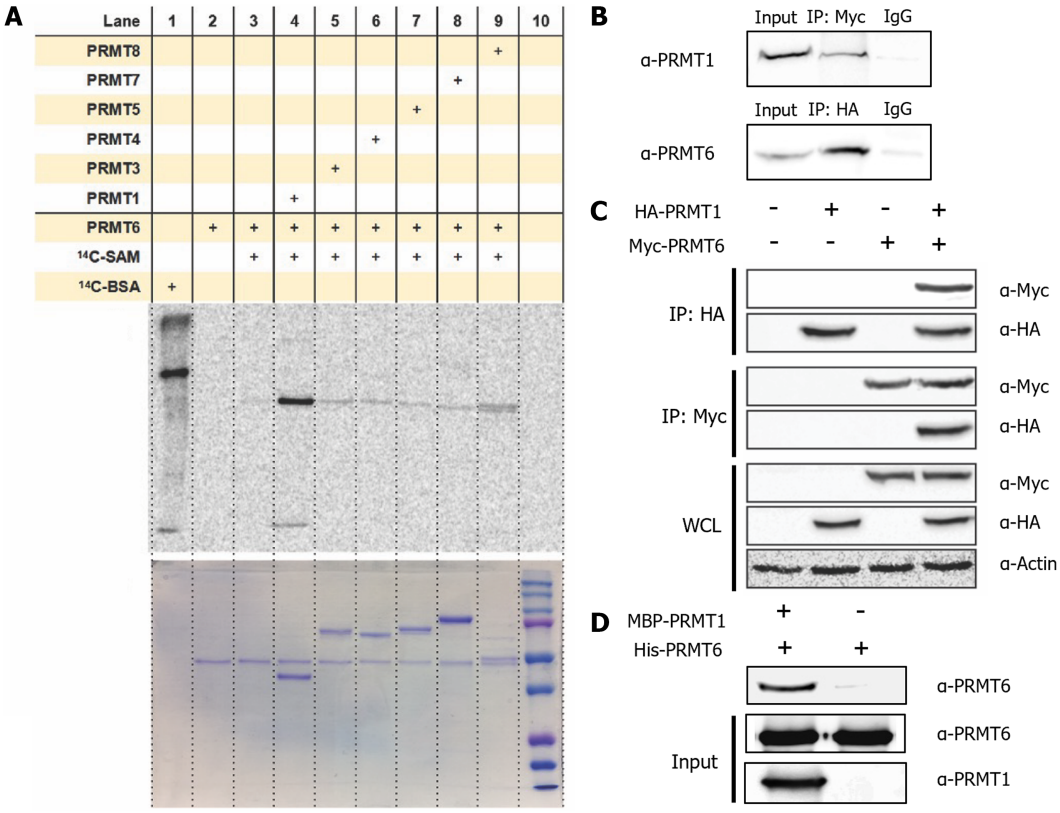
RESULTS

PRMT6 interacts with PRMT1

To investigate potential interactions between different PRMT members, PRMT6 was incubated with one of the other PRMT members in the presence of (^{3}H) SAM. The reaction mixture was resolved on SDS-PAGE and subjected to autoradiography imaging. We clearly observed PRMT6 underwent automethylation (Figure 1A), which coincides with the literature reports[40,54]. Of surprise, when mixed with PRMT1, the methylation of PRMT6 was significantly augmented (Figure 1A). This finding provided the initial evidence that PRMT1 interacted with PRMT6 and methylated the latter.

To validate the interaction between PRMT1 and PRMT6 in vivo, HEK293T cells were transfected with *HA-PRMT1* or *Myc-PRMT6*, the cell lysate was extracted after transfection. The anti-Myc antibody or anti-HA antibody was respectively bound to A/G agarose beads then the whole cell lysate was added. The proteins immunoprecipitated by the anti-Myc or anti-HA were eluted and western blot analysis was performed to detect PRMT1 or PRMT6. The result shown in Figure 1B clearly proved that the transfected Myc-PRMT6 immunoprecipitated endogenous PRMT1 and reciprocally, the transfected HA-PRMT1 immunoprecipitated endogenous PRMT6 in HEK293T cells. Moreover, in the HEK293T cells co-transfected with *HA-PRMT1*, *Myc-PRMT6* plasmids, *HA-PRMT1* and *Myc-PRMT6* were capable of reciprocally immunoprecipitated each other from the co-transfected cell lysate (Figure 1C).

To further verify a direct physical interaction exists between PRMT1 and PRMT6, MBP pull-down assay was performed in vitro using recombinant MBP-tagged PRMT1 protein. MBP-PRMT1 was incubated with amylose beads first and PRMT6 was incubated with the beads after washing. The resin was washed and protein was eluted by SDS-loading buffer. As shown in Figure 1D, PRMT6 was pulled down on MBP-PRMT1-bound amylose resin. Thus, this pull-down



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Figure 1 Protein arginine methyltransferase 1 interacts with protein arginine methyltransferase 6 in vitro and in vivo. A: Gel electrophoresis-autoradiographic image analysis of protein arginine methyltransferase (PRMT) 6 methylation incubated with other PRMT family members. 0.5 μM of PRMT6 was incubated with 1 μM of PRMT1, PRMT3, coactivator associated arginine methyltransferase (PRMT4), PRMT5, PRMT7 and PRMT8. Reactions were held for 30 min at 30 ℃, the samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% polyacrylamide gel; B: Co-immunoprecipitation assay was performed between HA-PRMT1 and endogenous PRMT6 or Myc-PRMT6 and endogenous PRMT1 in HEK293T cells. Co-transfected HEK293T cell lysate was immunoprecipitated with anti-HA antibody or anti-Myc antibody followed by protein A/G plus agarose beads, proteins on the beads were eluted and separated on 12% SDS-PAGE and were detected by anti-PRMT1 or anti-PRMT6 antibody; C: Immunoprecipitation assay was performed between HA-PRMT1 and Myc-PRMT6. Single transfected HEK293T Cell lysate was immunoprecipitated with anti-HA antibody or anti-Myc antibody followed by protein A/G plus agarose beads, proteins on the beads were eluted and separated on 12% SDS-PAGE and were detected by anti-Myc or anti-HA antibody; D: Western blot analysis of maltose-binding protein (MBP) pull down assay. PRMT6 binds to purified Amylose beads or MBP-PRMT1 was detected. PRMT1 was incubated with the amylose beads for 30 min, followed by washing by phosphate-buffered saline and incubated with PRMT6 for 4h. The resin was washed and the pulled-down proteins were eluted and separated by 12% SDS-PAGE and detected by anti-PRMT1 or anti-PRMT6. IgG: Immunoglobulin G; PRMT: Protein arginine methyltransferase; SAM: S-adenosyl methionine.

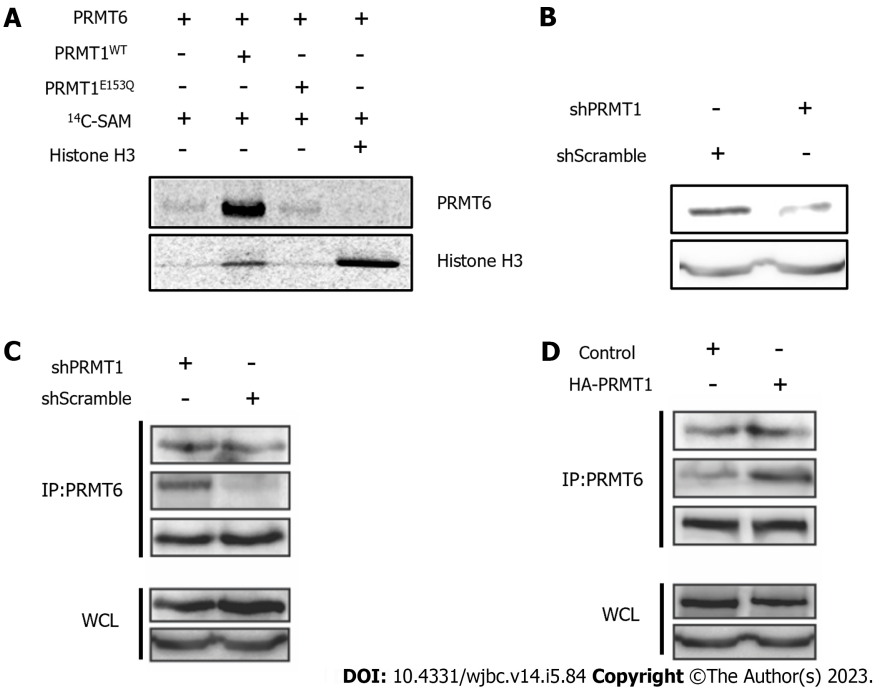
analysis supported that there was a direct interaction between PRMT6 and PRMT1.

PRMT6 is methylated by PRMT1

Having known that PRMT1 interacts with PRMT6 in vitro and in vivo, we next examined more details about the activity of PRMT1 in transferring methyl group (s) to PRMT6 by performing gel electrophoresis autoradiography. The inactive $PRMT1^{E153Q}$ was expressed and used a negative control to validate PRMT1 activity on PRMT6 methylation. As displayed in Figure 2A, PRMT6 itself in the presence of ^{14}C-SAM exhibited automethylation activity of PRMT6, confirming the result in Figure 1A. PRMT6 methylation band was much stronger in the presence of $PRMT1^{WT}$, but not the inactive E153Q mutant, clearly showing that PRMT6 was a substrate of $PRMT1^{WT}$. Notably, when histone H3, a substrate of PRMT6, was introduced, the automethylation of PRMT6 diminished significantly (Figure 2A), implying that PRMT6 prefers methylating its histone substrate rather than itself. To further verify PRMT6 methylation by PRMT1, we knocked down the PRMT1 level in HEK293T cells by shPRMT1 (Figure 2B) and then performed the immunoprecipitation of PRMT6. The result showed that both ADMA and MMA levels in PRMT6 was decreased as a result of PRMT1 knockdown (Figure 2C). Furthermore, we overexpressed PRMT1 in HEK293T cells and immunoprecipitated endogenous PRMT6. The immunoblot clearly showed an increase in the ADMA level in PRMT6 (Figure 2D). These biochemical and cellular assay data collectively demonstrated that PRMT6 was a substrate of PRMT1.

PRMT1-mediated methylation of PRMT6 goes much faster than PRMT6 automethylation

We then sought to further quantify PRMT1-catayzed PRMT6 methylation and compared this activity with PRMT6 automethylation. PRMT6 methylation level was checked at different time points in the presence of $PRMT1^{WT}$ (Figure 3A). At each time point, we observed that the existence of $PRMT1^{WT}$ led to an increasingly darker ^{14}C-labeled PRMT6



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Figure 2 Protein arginine methyltransferase 6 is methylated by protein arginine methyltransferase 1. A: Gel electrophoresis-autoradiographic image analysis of histone H3 methylation level change in the presence of protein arginine methyltransferase (PRMT) 1WT or PRMT1E153Q. 1 μM PRMT6 and 20 μM 14C-S-adenosyl methionine were incubated with or without 3 μM PRMT1WT, PRMT1E153Q or H3 protein respectively at 30 °C in the reaction buffer for 1h. The samples were separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis polyacrylamide gel. The radioactive SDS gel was dried and stored for two days and then scanned for the phosphor image; B: Western blot was performed to analysis PRMT1 level in HEK293T cells transfected by hPRMT1 or shScramble; C and D: Immunoprecipitation was performed to precipitate PRMT6 in HEK293T cell lysate transfected by shPRMT1 or shScramble or HA-PRMT1, followed by western blot to analysis monomethyl arginine and asymmetric dimethylarginine level of precipitated PRMT6. PRMT: Protein arginine methyltransferase.

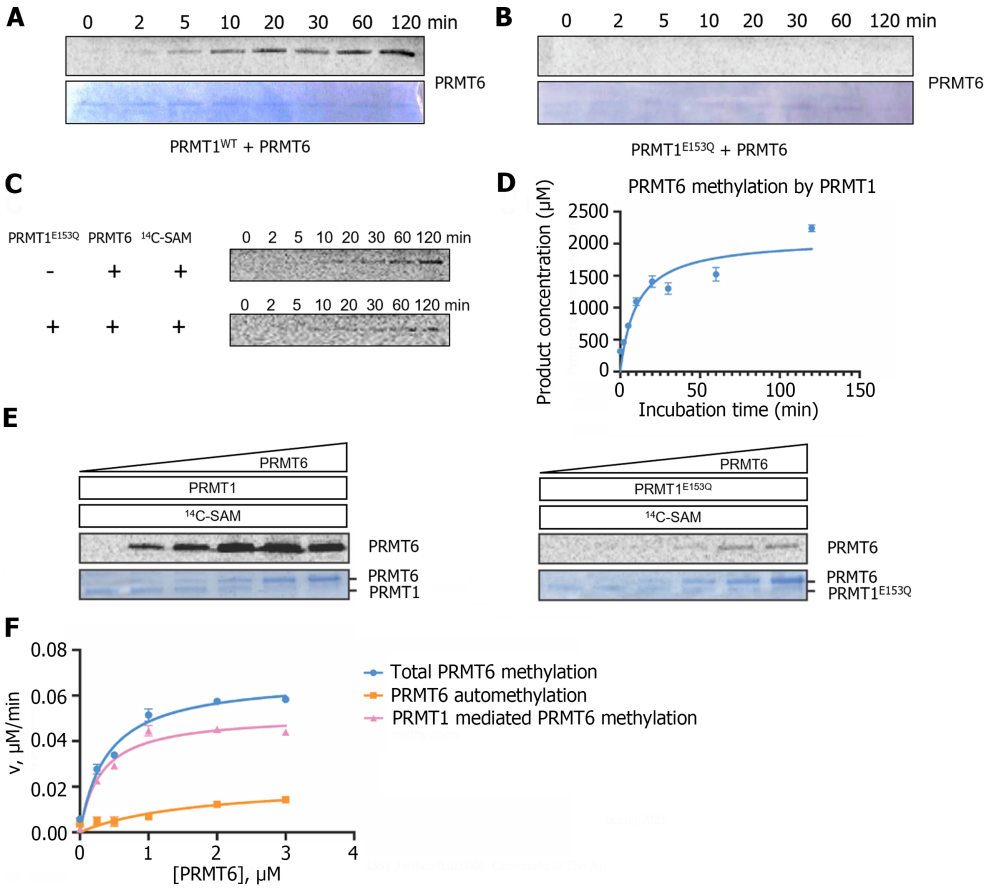
methylation band. In contrast, under the same condition, PRMT6 methylation level was barely invisible at different time points in the presence of PRMT1^{E153Q} which was the inactive form of PRMT1 (Figure 3B). This data further proved that the observed methylation of PRMT6 in Figure 1A was resulted from PRMT1 activity.

Although the automethylation band was not strong when the final concentration of PRMT6 was at 1.5 μM (Figure 3B), once the concentration of PRMT6 was increased to 4.5 μM, the methylation band became very clear when the incubation time was higher than 30 min (Figure 3C). In addition, the automethylation of PRMT6 appeared to be affected slightly by the addition of PRMT1^{E153Q} (Figure 3C). The quantification of the band intensities of Figure 3A was shown in Figure 3D. We also analyzed PRMT1-catalyzed PRMT6 methylation at different PRMT6 concentrations (Figure 3E). The quantification of band intensities was shown in Figure 3F. PRMT6 automethylation was subtracted from the total methylation to calculate PRMT1-catalyzed methylation. The data were fit with the Michaelis-Menton kinetic equation, yielding k_{cat} of 0.066 ± 0.009 min^{-1} and K_m of 0.3765 ± 0.06 μM. PRMT6 automethylation activity showed k_{cat} of 0.040 ± 0.006 min^{-1} and K_m of 0.1403 ± 0.05 μM. Clearly, PRMT6 methylation by PRMT1 is much stronger than PRMT6 automethylation.

The major methylation site in PRMT6 is R106 for PRMT1 catalysis

It is critical to know the residue site (s) in PRMT6 methylated by PRMT1 in order to understand the nature and consequence of this methylation event. Toward this goal, we incubated PRMT6 and SAM together with PRMT1, resolved the mixture on the SDS-PAGE gel, cut out the PRMT6 band and detected the modification sites by LC-MS/MS analysis. From the database search, PRMT6 was identified (Homo sapiens, Gene ID 55170, 41937.4 Da), with 29 unique peptides, 57 unique spectra, 462 total spectra, and 307/375 amino acids (82% coverage). Two arginine residues were detected to be dimethylated: Arg-82 and arg-174, and three arginine residues were detected to be mono-methylated: Arg-29, arg-106, and arg-228 (Figure 4A and B).

To confirm these methylation sites in PRMT6, we generated several mutants, PRMT6^{R29K}, PRMT6^{R82K}, PRMT6^{R106K}, PRMT6^{R174K} and PRMT6^{R228K}, by performing site-directed mutagenesis. A previous study showed that PRMT1 has two important conserved methionine residues and both PRMT1^{M48L} and PRMT1^{M155A} mutants showed an automethylation activity compared to wild-type PRMT1[55]. These results indicated that M48 and M155 are important for PRMT1 substrate recognition and relevant to the automethylation activity of mutant PRMT1. Thus, the two corresponding mutations in PRMT6, *i.e.* PRMT6^{M60L} and PRMT6^{M166A}, were also generated for the following tests. The mutants were expressed as his6x-tagged proteins in *Escherichia coli* (*E. coli*) BL21 (DE3) and purified with Ni-charged affinity chromatography. All the mutants were tested in the radioactive autography assay for detecting their methylation levels. Two sets of methylation tests, with and without PRMT1, were run in parallel, so that the automethylation can be subtracted from the total methylation to obtain the PRMT1-mediated methylation (Figure 5A and B).



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Figure 3 Time-dependent and concentration-dependent protein arginine methyltransferase 6 methylation catalyzed by protein arginine methyltransferase 1. A and B: Time-dependent radioactive gel methyltransferase assays were carried out at 30 °C in an incubation system of 30 μL. 1.5 μM of protein arginine methyltransferase (PRMT) 6 and 10 μM of 14C-S-adenosyl methionine (SAM) were incubated with 0.4 μM of PRMT1WT or PRMT1E153Q, and the reactions were quenched at different time points (0-120 min) by sodium dodecyl sulfate (SDS) loading buffer. The reaction mixture was separated on 12% SDS-polyacrylamide gel electrophoresis (PAGE) and methylated PRMT6 were visualized by Coomassie blue staining and phosphor image scanning, respectively; C: Gel electrophoresis autoradiography assays were performed at 30 °C in an incubation system of 30 μL. 4.5 μM PRMT6 was incubated with 10 μM 14C-SAM with or without 0.4 μM of PRMT1E153Q; D: Time course of PRMT6 methylation with PRMT1, the linear part of the curve was used to calculate the steady-state rate; E: Concentration-dependent radioactive gel methyltransferase assay was performed. Different concentration of PRMT6 (0, 0.25, 0.5, 1, 2, 4 M) was incubated with 10 M 14C-SAM in the presence of 0.2 M of PRMT1WT or PRMT1E153Q in the reaction buffer at 30 °C for 1h. The reaction mixture was separated on 12% SDS-PAGE and the phosphor image was quantified by ImageJ. Data were normalized according to the reading of 5 M 14C-BSA and the reaction yield was calculated according to the reading of 5 M reaction mixture in liquid scintillation; F: Comparison of the total PRMT6 methylation in the presence of PRMT1, PRMT6 automethylation, and PRMT1-mediated PRMT6 methylation at different concentration of PRMT6. SAM: S-adenosyl methionine; PRMT: Protein arginine methyltransferase.

Compared to wt-PRMT6, PRMT6^{R106K} mutant showed significantly weaker methylation by PRMT1 (-23% methylation left) but still underwent automethylation. On the other hand, PRMT6^{R174K} mutant retained -80% PRMT1-catalyzed methylation but showed little automethylation activity (-4% methylation left). This indicates that R106 is likely the major targeting site for PRMT1, but not a major PRMT6 automethylation site. On the other hand, R174 may be the major automethylation site in PRMT6, but not a major PRMT1-mediated methylation site. It might also be possible is that R174 was critical for PRMT6 catalysis and thus R174K mutation compromised its enzymatic activity. PRMT6^{R82K}, unexpectedly, showed equal PRMT1-catalyzed methylation and even higher automethylation. In addition, M60L methylation by PRMT1 was lowered to -33% and M166A automethylation was decreased to -38%, indicating their moderate roles in these two types of methylation processes.

Methylation by PRMT1 regulates the enzymatic activity of PRMT6

To determine the possible effect of arginine methylation on PRMT6 by PRMT1, selected mutants were tested for their differences in methyltransferase activity on H3 (1-31) peptide. The Michaelis-Menton kinetic curves and catalytic parameters for each mutant were compared in Figure 6A and Table 1. PRMT6^{WT} had a k_{cat} of 0.0063 ± 0.0006 min^{-1} and a K_m of 8.4 ± 0.7 μM. Notably, PRMT6R^{106K} which was a poor substrate of PRMT1 showed -2-fold increase in turnover rate (k_{cat} = 0.0122 ± 0.0004 min^{-1}) compared with PRMT6^{WT}. This raised the possibility that PRMT1-mediated methylation on R106 suppressed the activity of PRMT6. To confirm the effect of PRMT1 on PRMT6 activity, radioactive gel methylation assay was performed with PRMT6 being incubated with histone H3 and varying concentrations of PRMT1^{WT}. The result showed that as the PRMT1 amount increased, the PRMT6 methylation level increased while histone H3 methylation level decreased gradually (Figure 6B). Additionally, we analyzed the level of H3R2 methylation, a key target of PRMT6, in

Table 1 Steady-state kinetic parameters of protein arginine methyltransferase 6 mutants

PRMT6 mutant	k_{cat} (min^{-1})	K_m (M)	k_{cat}/K_m (M^{-1} min^{-1})
WT	0.0063 ± 0.0006	8.4 ± 0.7	0.00075 ± 0.00009
R106K	0.0122 ± 0.0004	9.9 ± 1.2	0.0012 ± 0.00015
R174K	N/A	N/A	N/A
M60L	0.0017 ± 0.0002	10.0 ± 3.5	0.00017 ± 0.00006
M166A	0.00031 ± 0.00003	40 ± 19	0.0000077 ± 0.0000037

PRMT6: Protein arginine methyltransferase 6. N/A: Not applicable.

HEK293T cells transfected with the HA-PRMT1 plasmid. Immunoblotting of the cell lysate showed that H3R2me2a level was appreciably lower in the PRMT1 overexpressed cells (Figure 6C). These data together demonstrated that PRMT1-mediated methylation of PRMT6 suppressed the enzymatic activity of PRMT6 on H3 substrate. Notably, $PRMT6^{R174K}$ gave no detectable activity on H3 methylation which means R174 is critical for PRMT6 catalytic activity and coincides with that R174K mutation abolishes its automethylation activity as well (Figure 6A). $PRMT6^{M60L}$ and $PRMT6^{M166A}$ mutations also lowered the activity of PRMT6 significantly. Overall, methylation of PRMT6 at the major site R106 induced by PRMT1 negatively regulates PRMT6 enzymatic methyltransferase activity.

Inspired by the previous work which had demonstrated the interaction between PRMT1 and PRMT2 affects PRMT1 enzymatic activity, we wondered if the interaction between PRMT1 and PRMT6 affects PRMT1 enzymatic activity or not. For this purpose, PRMT1 was incubated with inactive $PRMT6^{VLD:KLA}$ mutant, and then histone H4 was added. As showed in Figure 6D, H4 methylation band intensity was constant as PRMT6 concentrations were varied. Thus, PRMT6 did not affect PRMT1 activity.

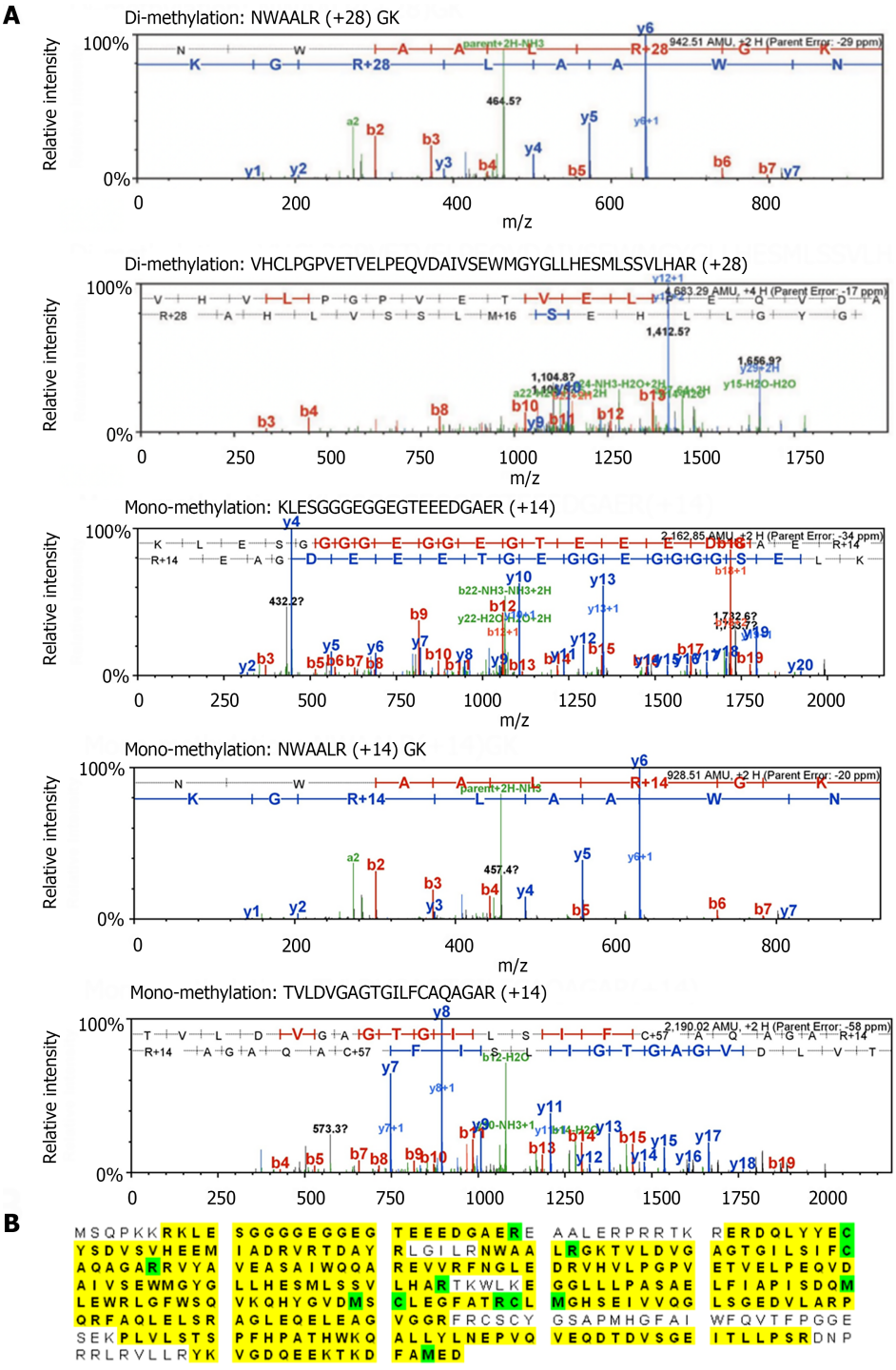
DISCUSSION

PRMT enzymes interact with various cellular proteins to regulate biological processes such as gene transcription, DNA repair, and RNA splicing and signal transduction[10,56]. In particular, a few studies have shown the probability that one PRMT interacts with another member in the PRMT family, forming heterological complexes. Pak *et al*[50] demonstrated the formation of a heterodimer between PRMT1 and PRMT2, which increases PRMT1 enzymatic activity. Chen *et al*[53] showed that PRMT5 is an interactive partner of PRMT6, and PRMT6 functionally associates with PRMT5 to promote the progression of colorectal cancer by downregulating the expression of *CDKN2B* and *CCNG1*. A recent study showed that CARM1 interacts with PRMT5 and PRMT5 is methylated by CARM1 at R505[52]. In the current work, we found that PRMT1 interacts with PRMT6 and methylates PRMT6. In the course of our studies, a recent study by Schneider *et al*[57] found that PRMT1 was co-precipitated with PRMT6, which is in good agreement with this work. The intricate interplay between PRMTs could be a common biochemical strategy that eukaryotic cells utilize to fine tune enzymatic activities of these enzymes for arginine methylation control on the global scale as well as on specific proteins.

In our immunoprecipitation experiments, PRMT1 and PRMT6 were reciprocally pulled down from the co-transfected as well as single-transfected cells. The interaction between PRMT1 and PRMT6 hint at that multiple heterological interactions may exist between different PRMT isoforms. When using eukaryotic systems to express PRMTs, caution should be taken that one or more other PRMT family members may be co-eluted, complicating the subsequent enzymatic activity analysis.

A few PRMT family members have been demonstrated to undergo automethylation, including PRMT1 mutants M48L and M155A, CARM1, PRMT6, and PRMT8[40,56,58,59]. Among these PRMTs, PRMT6 has been reported to methylate itself at site R35[40]. In our study, PRMT6 methylation level was significantly enhanced by the addition of PRMT1. To substantiate PRMT1-catalyzed PRMT6 methylation, we compared PRMT6 total methylation with the automethylation. The presence of $PRMT1^{WT}$ rather than the inactive $PRMT1^{E153Q}$ mutant significantly enhanced the methylation level of PRMT6, demonstrating that PRMT6 is a novel substrate of PRMT1. Our quantitative catalytic rate measurements showed that 0.4 mM PRMT1 was able to accelerate the methylation rate of 5 mM PRMT6 by ~4 fold. It is conceivable to speculate that PRMT1 may be largely responsible for the observed PRMT6 methylation in mammalian cells wherein PRMT1 acts as the predominant arginine methyltransferase *in vivo*. PRMT1-catalyzed PRMT6 methylation showed a K_m of 1.5 ± 0.6 mM, comparable to that of PRMT1-H4 interaction (K_m = 1.69 ± 0.39 mM)[60].

LC-MS/MS analysis of methylated PRMT6 by PRMT1 exhibited high sequence coverage and identified five target arginine residues. Among those residues, R106 was found as the major PRMT1-mediated methylation site through the site-directed mutagenesis study (Figure 5B). Additionally, R29 seemed to be slightly methylated by PRMT1. However, no significant changes in PRMT1-mediated methylation were observed for R82K, R174K and R228K. Notably, R106 was not the automethylation site since $PRMT6^{R106K}$ still retains similar automethylation level as the $PRMT6^{WT}$. The steady-state kinetic measurement showed no detectable methylation activity of R174K on the H3 peptide (Figure 6). This suggests that either the R174 residue or methylation of R174 is essential for PRMT6 activity. A previous study showed that PRMT6 automethylation occurred at R29, R35, and R37, with R35 confirmed as the automethylation site regulating PRMT6

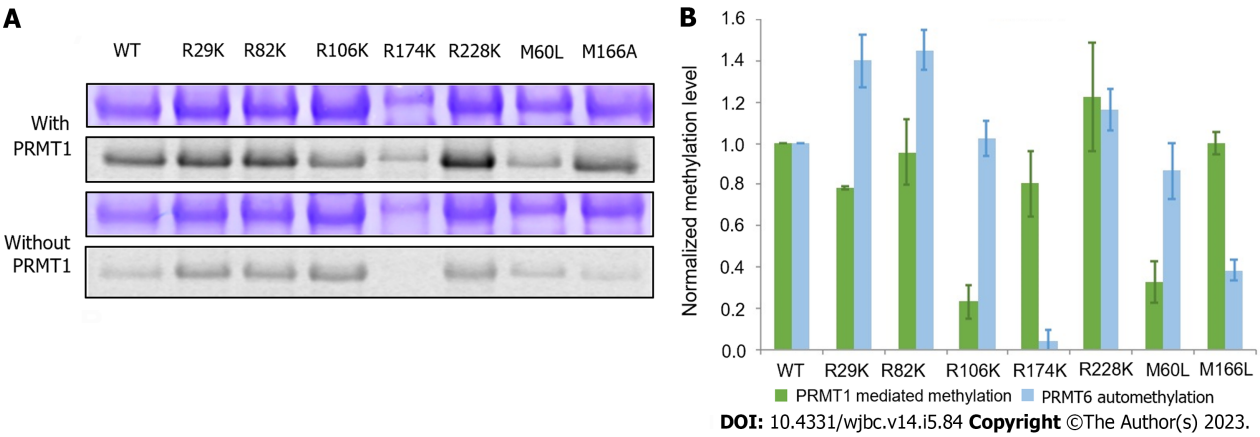


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Figure 4 Liquid chromatography with tandem mass spectrometry analysis of the methylation sites on protein arginine methyltransferase 6. A: 5.5 M protein arginine methyltransferase (PRMT) 6 and 200 M S-adenosyl methionine were incubated with 1 M PRMT1 at 30 ℃ for 3 h in the reaction buffer. The methylated PRMT6 band was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, cut from the gel, washed and vacuum-dried. The sample was sent to UAB proteomics and mass spectrometry facility (Birmingham, AL 35294) for in-gel digestion and liquid chromatography-tandem mass spectrometry (MS/MS) detection. Tandem MS spectra showing the arginine-dimethylated peptides; B: PRMT6 protein sequence coverage (yellow) and the modified residues (green) detected by tandem MS analysis. Green "C" is with carboamidomethylation, and green "M" is with oxidation.

stability[61]. However, in our study, R35 was not identified as a methylation site by LC-MS/MS. Nevertheless, we showed that R29 is not a major methylation site since PRMT6R29A still exhibited similar automethylation level as PRMT6^{WT} and R29 was still efficiently methylated by PRMT1. Further investigation is required to determine the other automethylation site (s) on PRMT6 and their effects.

According to the sequence alignment, PRMT1 and PRMT6 have reasonably high sequence similarity (54% positives). The corresponding sites of R82, R106, R174, R228 in PRMT6 are conserved as K70, R94, R163, K215 in PRMT1, respectively (Figure 7). R29 is on the flexible N-terminal tail of PRMT6. It is interesting to see that R106 and R174 of



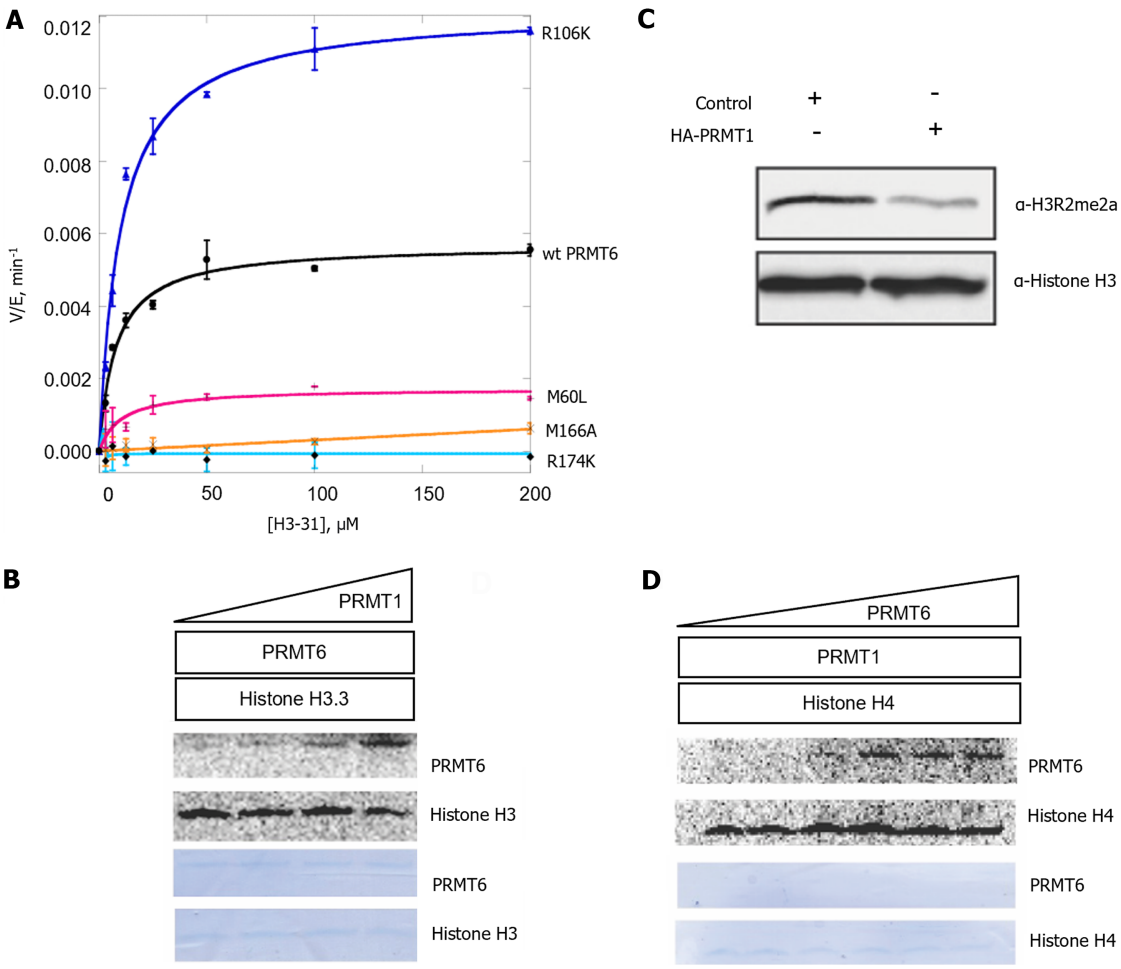
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Figure 5 Comparison of protein arginine methyltransferase 1-mediated methylation and automethylation among different protein arginine methyltransferase 6 mutants. A: Radioactive methyltransferase assay was performed to compare protein arginine methyltransferase (PRMT) 1-mediated methylation level and automethylation level of PRMT6 mutants. 5 M of each PRMT6 mutant was incubated with 30 M 14C-S-adenosyl methionine in the presence or absence of 1 M PRMT1 in the reaction buffer at 30 ℃ for 2 h. The products were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized by Coomassie blue staining and phosphor image scanning. The methylation intensity of each mutant was quantified with Quantity One and normalized against PRMT6WT. The assay was repeated at least three times. Coomassie blue staining and phosphor image of methylated PRMT6 mutants in the presence or absence of PRMT1; B: Column graph comparing the methylation of PRMT6 m. PRMT: Protein arginine methyltransferase.

PRMT6 are methylated but methylation of the corresponding residues R94 and R163 of PRMT1 are not found. In the crystal structure of PRMT6, R106 (aligned with R94 of PRMT1) is located on the surface loop connecting helix A and strand 2 of the SAM-binding domain. The flexibility of this loop region may make R106 of PRMT6 accessible to the active site of PRMT1. R174 (aligned with R163 of PRMT1) is located on Helix D and faces the interface between the SAM-binding domain and the barrel-like domain. Likely R174 on PRMT6 is important for maintaining the α-helix structure which is critical for catalysis.

From a biochemical perspective, methylation of arginine residues can introduce steric hindrance of the guanidino group and compromise its H-bonding capacity, thus altering protein local structure or protein-protein interaction or regulating the enzymatic activity. PRMT6-R106K, which significantly lost PRMT1-mediated methylation compared to PRMT6-WT, showed a 2-fold increase in its catalytic activity (Figure 6), highlighting that PRMT1-mediated methylation suppresses the enzymatic activity of PRMT6. Indeed, the quantitative biochemical assays showed that PRMT1-mediated PRMT6 methylation significantly decreased the enzymatic activity of PRMT6 on its substrate histone H3. Further, cellular assays showed that PRMT1 overexpression suppressed H3R2 methylation, an important cellular target of PRMT6. Interestingly, a previous study showed that PRMT6 automethylation is important for its stability and its activity which is involved in inhibiting HIV-1 replication[54]. Therefore, PRMT1-mediated PRMT6 methylation and PRMT6 itself-mediated automethylation have opposite effects on PRMT6 activity. Our results support that PRMT1 counteracts both PRMT6 automethylation activity and its histone substrate methylation activity. This observation may be physiologically significant. It is known that PRMT1 activates transcription but PRMT6 represses transcription[37]. Based on our finding, we propose that PRMT1 regulates gene transcription on the chromatin through dual mechanisms: Direct action of histone methylation and repressive methylation of PRMT6. Thus, PRMT1's impact on transcription extends beyond its primary histone methylation function, fine-tuning gene expression is further achieved by counteracting the activity of a repressive transcriptional cofactor, *i.e.* PRMT6. The previous research has identified a number of gene targets regulated by PRMT6. For instance, PRMT6 downregulates tumor suppressor genes p53, p21, p16 and p27 through the generation of H3R2me2a marks[61-64]. PRMT6 is capable of downregulating *HOXA* genes[65]. In the U2OS cells, PRMT6 is shown to repress the expression of TSP-1[32]. Furthermore, the presence of the H3R2me2a mark impedes the recognition of the H3K4me3 modification by reader proteins[38]. Of great interest, Choi *et al*[66] recently observed the suppressive effect of PRMT1 on PRMT6 in skeletal muscle and myoblasts, which is well in line with our results.

In conclusion, this current investigation presented a new finding about the interaction between two important PRMT members PRMT1 and PRMT6, and intriguingly, PRMT6 was shown to be a novel substrate of PRMT1. We identified specific arginine residues of PRMT6 that are methylated by PRMT1, with R106 being the major methylation site. Combined biochemical and cellular data showed that PRMT1 downregulates the enzymatic activity of PRMT6 in histone H3 methylation. Understanding the intricate interplays between PRMT1 and PRMT6 will be crucial for deciphering the complex regulatory networks governing gene expression. Future research is warranted to elucidate whether and how various signaling pathways regulated by PRMT6 can be counteracted by PRMT1. Priority should be given on the examination of functional consequences of PRMT6 methylation mediated by PRMT1 on different protein substrates and in different biological contexts.

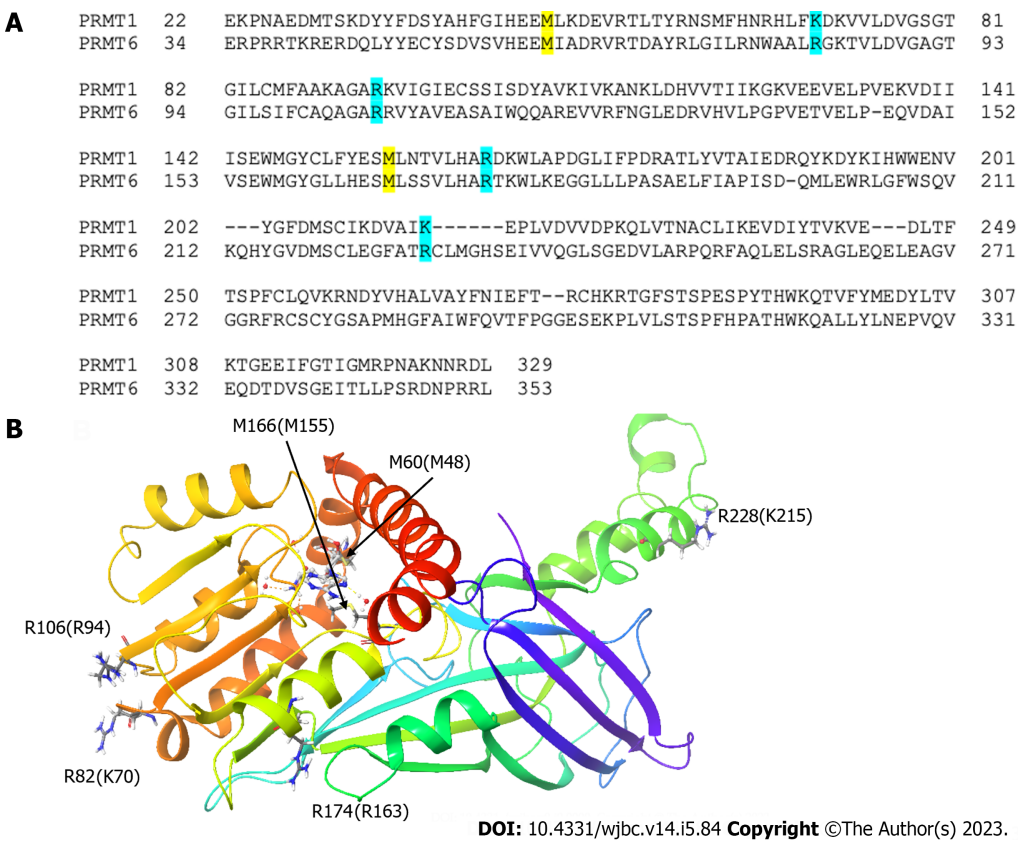


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Figure 6 Methylation by protein arginine methyltransferase 1 may regulate the enzymatic activity of protein arginine methyltransferase 6. A: Radioactive methyltransferase assay was performed to show activity of protein arginine methyltransferase (PRMT) 6 wild type and PRMT6 mutants on histone H3 (1-31). 0.6 M PRMT6 mutant and 30 μM 14C-S-adenosyl methionine (SAM) were incubated with 0 to 200 μM of H3 (1-31). After the enzyme was added, the reaction was continuously incubated for 60 min and then quenched by spreading the mixture onto P81 filter paper. Following washing and drying the paper, liquid scintillation was performed to quantify the methylated products. The data were fit to the Michaelis-Menten curve; B: Radioactive methyltransferase assay was performed to show histone H3 methylation level change as PRMT1 concentration change. Different concentration of PRMT1 was incubated with 30 μM 14C-SAM and 1 μM PRMT6 for 1 h, histone H3 was added and incubated for another 1 h. The systems were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and the phosphor image was captured after 72 h; C: Histone H3 methylation of HEK293T cell lysate was detected by western blot using anti-H3R2me2a antibody. HEK293T cell was transfected with HA-PRMT1 or control and cell lysate was extracted; D: Radioactive methyltransferase assay was performed to show histone H4 methylation level change as PRMT6 concentration change. Different concentration of PRMT6 was incubated with PRMT1 and 30 μM 14C-SAM, then 1 μM histone H4 was added and incubated for another 1 h. The systems were separated by 12% SDS-PAGE gel and the phosphor image was captured after 72 h. PRMT: Protein arginine methyltransferase.

CONCLUSION

In conclusion, this current investigation presented a new finding about the interaction between two important PRMT members PRMT1 and PRMT6, and intriguingly, PRMT6 was shown to be a novel substrate of PRMT1. We identified specific arginine residues of PRMT6 that are methylated by PRMT1, with R106 being the major methylation site. Combined biochemical and cellular data showed that PRMT1 downregulates the enzymatic activity of PRMT6 in histone H3 methylation. Understanding the intricate interplays between PRMT1 and PRMT6 will be crucial for deciphering the complex regulatory networks governing gene expression. Future research is warranted to elucidate whether and how various signaling pathways regulated by PRMT6 can be counteracted by PRMT1. Priority should be given on the examination of functional consequences of PRMT6 methylation mediated by PRMT1 on different protein substrates and in different biological contexts.



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Figure 7 The mutation sites of protein arginine methyltransferase 6 and corresponding residues on protein arginine methyltransferase 1. A: Sequence alignment of protein arginine methyltransferase (PRMT) 1 and PRMT6 showing the corresponding mutation sites; B: The corresponding mutation sites are highlighted on PRMT6 structure. hPRMT6 monomer (PDB: 4Y30) is shown in a cartoon model. The mutation sites of PRMT6 with their corresponding residues on PRMT1 and the S-Adenosyl-L-homocysteine molecule are shown in a stick model.

ARTICLE HIGHLIGHTS

Research background

Post-translational modifications play key roles in various biological processes. Protein arginine methyltransferases (PRMTs) transfer the methyl group to specific arginine residues. Both PRMT1 and PRMT6 have emerges as crucial factors in the development and progression of multiple cancer types. We posit that PRMT1 and PRMT6 might interplay directly or in-directly in multiple ways accounting for shared disease phenotypes.

Research motivation

The tumor-promoting roles of PRMT1 and PRMT6 in oncology are executed through their methylation of histone and non-histone substrates. Expression profile analysis suggests that PRMT1 and PRMT6 are both involved in multiple pathways which are fundamental for cell proliferation. We posit that PRMT1 and PRMT6 might interplay directly or in-directly for shared disease phenotypes. Thereby, neutralizing these enzymes' activities or their interactions may interfere with tumor progression and lead to anticancer beneficial effects.

Research objectives

Understanding the role and function of PRMTs and how they interact with each other, identification of the methylation site (s) on PRMT6. Knowing the mechanism of this intercommunication will help develop inhibitor of tumor progression and increase cancer therapeutic effect. Knowing the interaction between PRMT1 and PRMT6 regulate the enzymatic activity of PRMT6.

Research methods

Gel electrophoresis autoradiography was performed to test the methyltranferase activity of PRMTs and characterize the kinetics parameters of PRMTs. Liquid chromatography-tandem mass spectrometry analysis was performed to detect the PRMT6 methylation sites.

Research results

In this study we investigated the interaction between PRMT1 and PRMT6, and PRMT6 was shown to be a novel substrate of PRMT1. We identified specific arginine residues of PRMT6 that are methylated by PRMT1, with R106 being the major

methylation site. Combined biochemical and cellular data showed that PRMT1 downregulates the enzymatic activity of PRMT6 in histone H3 methylation.

Research conclusions

PRMT6 is methylated by PRMT1 and R106 is a major methylation site induced by PRMT1. PRMT1 methylation suppresses the activity of PRMT6.

Research perspectives

Future research is warranted to elucidate whether and how various signaling pathways regulated by PRMT6 can be counteracted by PRMT1. Priority should be given on the examination of functional consequences of PRMT6 methylation mediated by PRMT1 on different protein substrates and in different biological contexts.

FOOTNOTES

Author contributions: Cao MT, Feng Y and Zheng YG designed the research study; Cao MT and Feng Y performed the experiments; All authors analyzed the data, wrote the manuscript, and have read and approve the final manuscript.

Supported by National Institutes of Health, No. 5R01GM126154 and No. 1R35GM149230.

Institutional review board statement: The study was reviewed and approved by the University of Georgia Institutional Biosafety Committee protocol number.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

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S-Editor: Qu XL
L-Editor: A
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