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FRONTIER

Helicobacter pylori infection: How does age influence the inflammatory pattern?

Glauber Rocha Lima Araújo, Hanna Santos Marques, Maria Luísa Cordeiro Santos, Filipe Antônio França da Silva, Breno Bittencourt da Brito, Gabriel Lima Correa Santos, Fabrício Freire de Melo

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Abstract

The inflammatory pattern during *Helicobacter pylori* (*H. pylori*) infection is changeable and complex. During childhood, it is possible to observe a predominantly regulatory response, evidenced by high concentrations of key cytokines for the maintenance of Treg responses such as TGF-β1 and IL-10, in addition to high expression of the transcription factor FOXP3. On the other hand, there is a predominance of cytokines associated with the Th1 and Th17 responses among *H. pylori*-positive adults. In the last few years, the participation of the Th17 response in the gastric inflammation against *H. pylori* infection has been highlighted due to the high levels of TGF-β1 and IL-17 found in this infectious scenario, and growing evidence has supported a close relationship between this immune response profile and unfavorable outcomes related to the infection. Moreover, this cytokine profile might play a pivotal role in the effectiveness of anti-*H. pylori* vaccines. It is evident that age is one of the main factors influencing the gastric inflammatory pattern during the infection with *H. pylori*, and understanding the immune response against the bacterium can assist in the development of alternative prophylactic and therapeutic strategies against the infection as well as in the comprehension of the pathogenesis of the outcomes related to that microorganism.

Key Words: *Helicobacter pylori*; Inflammation; Treg response; Th1 response; Th17 response; Gastric diseases

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Core Tip: *Helicobacter pylori* (*H. pylori*) is a bacterium acquired mainly in childhood that increases the risk of developing certain gastric diseases. However, the main complications are noticed predominantly in adulthood. This can be explained based on the gastric inflammatory pattern against the pathogen, which changes as long as the infected individual gets older, favoring, during childhood, the persistence of the infection and then, in adulthood, the gastric damage. This article discusses the factors that can influence the gastric inflammatory pattern in individuals infected with *H. pylori*.

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BIOGRAPHY

Fabrício Freire de Melo (Figure 1), PhD, is a professor at the Multidisciplinary Institute of Health of the Universidade Federal da Bahia, Brazil. He is currently a Research and Extension Coordinator at the aforementioned institute. He holds a bachelor's degree in Biological Sciences from the Pontifícia Universidade Católica de Minas Gerais (2004), in Brazil, and a master's degree (2007), a PhD (2011), and a postdoctoral fellowship (2013) in Biological Sciences (Microbiology) from the Universidade Federal de Minas Gerais (UFMG), Brazil. Moreover, he was a visiting researcher at the Medical Entomology Laboratory at the René Rachou Institute, Fiocruz, Brazil.

He divides his professional activity between research work and academic teaching. His main research areas include *Helicobacter pylori* infection, arboviruses, and, currently, *SARS-CoV-2*. He has developed extensive work on *Helicobacter pylori*, which includes investigations on the differences between the immune responses observed in children and adults infected with the bacterium. His work in several areas has already been recognized and awarded worldwide, being the cover of the *World Journal of Clinical Oncology* (Volume 11, Issue 11).

He is a member of the editorial board of the *World Journal of Clinical Oncology*, an academic editor of the *World Journal of Gastroenterology*, and a reviewer for journals including the *World Journal of Gastroenterology*, *World Journal of Clinical Cases*, and *World Journal of Gastrointestinal Oncology*.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a microaerophilic, Gram-negative, rod-shaped, mobile bacterium of great clinical importance that is able to colonize the extremely hostile stomach environment[1].

Studies analyzing populations suggest that approximately 50% of the world population are infected with *H. pylori*. In addition, most *H. pylori* infections appear to be acquired during childhood, and estimates suggest that a third of the child population are or will be infected with the bacterium[2,3].

H. pylori infection is associated with the development of peptic ulcer and gastric cancer (GC), and the interactions between the virulence factors of the pathogen and the host immune response seem to be crucial in the development of those diseases[1,4]. Reviews show that 10% of those infected with *H. pylori* develop a peptic ulcer and 1%-3% develop GC[3]. *H. pylori* is a Group I carcinogen according to the International Agency for Research on Cancer (IARC), with 89% of all gastric cancers being attributable to this infection[2].

The host immune response to *H. pylori* is complex and changeable. It is possible to notice during childhood a predominantly regulatory inflammatory pattern (Treg), with higher concentrations of TGF-β1 and IL-10 than colonized adults, in addition to the greater number of FOXP3$^+$ Treg cells observed in the gastric mucosa of children. This predominantly regulatory pattern makes the gastric mucosa of children more

Figure 1 Fabrício Freire de Melo, PhD, Professor at the Universidade Federal da Bahia - Campus Anísio Teixeira, Brazil.

vulnerable to *H. pylori* colonization, but with milder inflammation when compared to what occurs in the mucosa of infected adults. As a result, the immune system of pediatric patients is not able to eliminate the *H. pylori* infection, and the bacterium persists in the gastric environment if left untreated. Moreover, damage to the gastric mucosa is less frequent during childhood, despite persistent mucosal colonization[5-8].

In adults, there is a predominant Th1 response, with higher levels of IFN-γ and IL-12p70 in the gastric mucosa, in contrast to the predominance of the regulatory response found during childhood. Besides, adults have a more intense Th17 response when compared to children. This can be verified by the higher mucosal concentrations of cytokines such as IL-17A and IL-23 and lower concentrations of TGF-β1, which, despite participating in the Treg response, when expressed at lower levels, seems to synergize with IL-6, promoting the expression of IL-23 receptors (IL-23r) and favoring an intense Th17 response. This cytokine profile is closely associated with the occurrence of damage to the gastric epithelium. Therefore, adults have a higher susceptibility to developing peptic ulcers, gastric atrophy, and intestinal metaplasia, a well-known precancerous lesion[5,6,9].

Of note, an increase in pro-inflammatory cytokines such as TNFα, IL-1α, IL-1β, IL-6, IL-2, and IL-17A is observed in *H. pylori*-positive children compared to *H. pylori*-negative infants. However, the Treg profile seems to overcome the inflammatory responses promoted by Th1 and Th17 cytokines in those individuals. This predominance of a regulatory immune response observed in infants might favor the colonization and persistence of the infection in the gastric mucosa, whereas the Th1 and Th17 responses induce a higher inflammatory activity in adults, leading to a higher risk of *H. pylori*-related gastric damage.

PREVALENCE

H. pylori infect about 4.4 billion people worldwide[2]. The prevalence of the infection is variable around the world and has changed over the last few years, with a notable reduction of the *H. pylori*-infected population, especially in developed countries[2,10-12]. Hooi *et al*[2] showed, through a meta-analysis, that the seroprevalence is higher in underdeveloped regions, and the highest prevalences were found in Africa (79.1%), Latin America and the Caribbean (63.4%), and Asia (54.7%). Otherwise, developed regions such as North America (37.1%) and Oceania (24.4%) have lower prevalence rates[3].

The infection is mainly acquired during childhood, and this phenomenon is predominantly observed in countries with a higher prevalence of *H. pylori*-positive individuals[13-15]. Moreover, higher prevalences of *H. pylori* infection are associated with lower socioeconomic status, household overcrowding, and lower educational

levels[11]. Sex may also influence the risk of acquiring the infection. A higher prevalence of the disease is usually observed among male subjects than in females. This may be related to hormonal factors, especially estrogen, which stimulates the immune response, and to a lower exposure to environmental factors such as smoking among women[16,17].

Furthermore, the prevalence may vary based on ethnic groups: Indigenous people in most countries are more susceptible to being infected[2]; a study in the United Arab Emirates showed a higher *H. pylori* prevalence among Africans than in Asian and Arabic populations, and, despite living in similar conditions to other ethnic groups, Malays were notably less affected by *H. pylori* infection than other people in that country[18-20]. In another study, Jonaityte *et al*[21] found a decline in the seroprevalence of *H. pylori* among medical students from Lithuania, with seroprevalences of 51.7, 30.4, 26.3, and 14.2% in 1995, 2012, 2016, and 2020, respectively. Besides, Africa, Western Asia, and South America are the regions with the highest incidence of *H. pylori*, while Oceania, North America, and Western Europe have lower prevalences of the bacterium[2].

BACTERIAL DENSITY AND GASTRIC INFLAMMATION

Despite being able to colonize all regions of the stomach, *H. pylori* proliferates better in certain anatomical areas, and higher bacterial densities are found in the antrum and cardia. Many factors can be responsible for this difference, such as the different levels of acid production in each portion of the stomach. In this sense, the regions with slightly lower acidity (antrum and cardia) are the regions with the highest *H. pylori* density[22,23].

Margarida *et al*[24], when studying 21 children infected with *H. pylori*, found infiltration of mononuclear (MN) cells in 50% of the cases. Furthermore, they did not find any neutrophil infiltrate in 40% of the participants, and, in 60% of the individuals, there was a slight eosinophilic infiltrate. Moreover, they have also found a relationship between bacterial density and MN and neutrophil cell counts in the stomach. Besides, they concluded that the infiltration of MN cells and neutrophils is lower in children infected with *H. pylori* than in *H. pylori*-negative adults. These findings were probably due to the differences between the immune response profiles predominating in each age group[2,9]. Thus, it is evident that the host immune response directed to the *H. pylori* can be influenced by several factors such as age and bacterial density, being complex and changeable.

CYTOKINE CONCENTRATIONS IN THE GASTRIC MUCOSA OF CHILDREN AND ADULTS

Given that *H. pylori* colonization is established mainly during childhood, that severer clinical outcomes related to the infection tend to occur as age advances, and that the immune system plays pivotal roles in *H. pylori*-related diseases, the following question is raised: Is the cytokine pattern observed in the immune response against the bacterium influenced by the age of colonized patients?

In an investigation enrolling Brazilian children and adults, our group has demonstrated that, among *H. pylori*-infected persons, infants tend to present a gastric Treg-polarized cytokine profile instead of the significant expression of Th17-related cytokines observed in older individuals. The analysis of the expression of cytokines in the gastric environment evidenced that IL-10 and TGF-β1 are expressed at higher levels in the former group, whereas the contrary was observed regarding the expression of IL-1β, IL-17A, and IL-23[5]. Those findings corroborate a precedent study by Harris *et al*, which showed more intense expression of the Treg-related cytokines TGF-β1 and IL-10 in children than in adults in a Chilean population[8]. Another study carried out by our group evaluated cytokines associated with innate and Th1 immune response in *H. pylori*-positive patients from various age groups[9]. We found that the gastric levels of IL-1α and TNF-α were significantly higher in children than in adults, whereas IL-2, IL-12p70, and IFN-γ were less expressed in infants than in older individuals (Figure 2). Interestingly, a drop in the gastric concentrations of IFN-γ and IL-12p70 in adults and an increase in the gastric mucosa levels of IL-1, IL-2, IL-12p70, and IFN-γ in children were observed with aging.

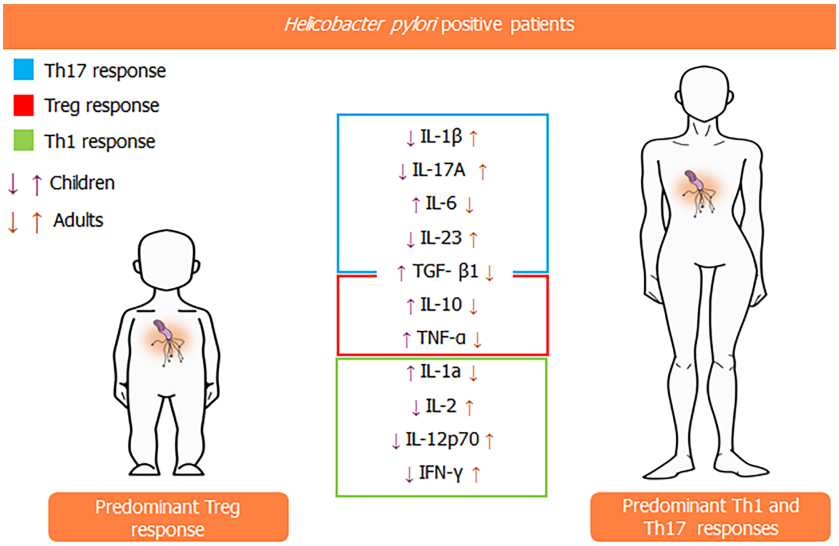

Figure 2 Comparison between gastric cytokines levels in children and adults.

Taken together, the aforementioned results show that age, indeed, influences the immune response against the bacterium and strongly suggest the occurrence of significant anti-inflammatory patterns among *H. pylori*-infected children, which might affect not only the development of gastric diseases but also other health-related aspects during the initial years of life. This hypothesis becomes even more relevant when considering that environmental stimuli are crucial for the development of the immune system in that life period, a position supported by the so-called hygiene hypothesis, which claims that the contact with microorganisms in early life is determinant for the maturation of the immune system[25]. Interestingly, a recent study by León *et al*[26] suggests that *H. pylori* may induce atopy modulation in children since they found that *H. pylori*-infected infants had higher expression of high-affinity IgE receptor (FcεRI) by peripheral dendritic cells and enhanced levels of FOXP3 and Latency Associated Peptide by T reg cells. The FcεRI is related to a regulatory dendritic cell profile since the interaction of IgE with that molecule fails to induce the maturation of these cells [27]. The possibility of systemic effects by *H. pylori* infection through the induction of immune system regulatory mechanisms makes us question the possible impacts of *H. pylori* eradication among children over the development of future immune system-related disorders. Although we understand and support the need for eliminating the bacterium, it has to be emphasized that this infection has been negatively correlated to the development of relevant immune system-linked diseases that are relevant among young people, including asthma[28]. In addition, the current scenario of widespread use of antibiotics and growing antimicrobial resistance among *H. pylori* strains should not be ignored[29]. Therefore, we hope that, along with the advances in the clinical analysis of genetic and epigenetic backgrounds, the future approaches to *H. pylori* infections and the decision on the necessity of bacterial eradication should be carried out in a more individualized manner, instead of the generalized, but necessary, treatments preconized by current guidelines.

Some studies have emphasized that immunizing agents against *H. pylori* should be able to induce a Th17 response to achieve satisfactory effectiveness. In that context, Velin *et al* induced mouse immunization using mucosally administered cholera toxin followed by *H. felis* challenge and observed that it induced a remarkable peak of CD4+IL-17+ T cells in the gastric mucosa[30]. Recently, a study by Chen *et al*[31] tried to immunize mice using a cyclic guanosine monophosphate-adenosine monophosphate as an adjuvant for the anti-*H. pylori* vaccine and observed that its effectiveness depended on high levels of antigen-specific Th1 and, mainly, Th17 responses. These findings draw attention to the aforementioned results showing low levels of Th17-related cytokines among *H. pylori*-infected children, which could represent an obstacle in the development of effective immunizing agents for that population. This is an important issue to be considered since the *H. pylori* infection is mainly acquired during childhood[31].

GASTRIC HISTOLOGY AND CYTOKINE CONCENTRATIONS

In our aforementioned study evaluating the variations of the Th1 immune response to the infection by *H. pylori* according to age, we observed that the increased levels of IFN-γ and IL-12p70 in the gastric environment were associated with an increase in MN cells in the gastric corpus and antrum. Moreover, when considering the group of young adults, IL-12p70 was linked to an increase in the count of both MN and PMN cells in the gastric antrum[9]. Interestingly, another study observed that the levels of IFN-γ and IL-12 were higher in infected children than in uninfected children ($P < 0.001$). In addition, these cytokines were positively correlated with the inflammation score ($P < 0.01$) and PMN infiltration, corroborating our findings[32]. In an analysis of polyclonal responses in $CD4^+$ T cells in *H. pylori*-positive children, a potent production of IFN-γ was also observed. However, the responses were stronger in adults, due to their higher frequency of memory T cells[33]. Curiously, some authors have observed that the levels of *IFN-γ* mRNA in infected children were lower when compared to infected adults[8,34]. These data suggest an increased regulatory response conducted by Treg cells in children, thus reducing the inflammatory Th1 response in the gastric mucosa[5,8]. In a recent prospective Brazilian study, it was observed that IL-27 is increased in individuals with *H. pylori*-related duodenal ulcer and absent in patients with GC. Moreover, higher gastric concentrations of IL-12p70 ($P < 0.001$) and IFN-γ ($P = 0.004$) were observed in patients with duodenal ulcers than in those with GC. In addition, IL-27 is positively correlated to the expression of IL-12p70, an important cytokine in Th1 responses that directly influences the pattern of inflammation in the antral mucosa of patients with duodenal ulcer[35]. The relationship between IL-12p70 and IFN-γ is well elucidated in the context of *H. pylori* infection. In a study that added neutralizing antibodies to IL-12 in gastric biopsy cultures, authors observed a negative regulation of signal transducer and activator of transcription 4 (STAT4), an important factor for the production of IFN-γ, leading to a significant decrease in the concentrations of this cytokine ($P < 0.001$)[36]. Therefore, considerable progress has been achieved in the understanding of these important interplays between cytokine variations between different age groups and among regions of the gastric mucosa. Although the presence of MN and PMN cell infiltration associated with Th1 responses has been described, further studies are needed to aid in the understanding of the dynamics and frequency of these cells in the context of the *H. pylori*-induced gastric diseases.

As aforementioned, the *H. pylori* gastric environment colonization leads to a polarization toward Th1/Th17 responses, whereas Treg cells are responsible for the induction of anti-inflammatory responses. Of note, the Treg cells can be divided into IL-10-secreting Tr1 cells, TGF-β1-producing Tr3 cells, and FOXP3-expressing CD4+CD25high Treg cells[37]. The latter cells seem to be crucial in the setting of *H. pylori* infection. As long as they suppress the immune response against the bacterium, the pathogen persistence in the gastric mucosa might be favored. In that context, when evaluating the host immune response against *H. pylori* in adults and children, our group found that the expression of $FOXP3^+$ Treg cells was significantly higher in the antrum of *H. pylori*-positive patients than in *H. pylori*-negative individuals[38]. This finding corroborates a previous study by Kandulski *et al*[39], which reported that *H. pylori* infection leads to a remarkable proportional enhancement of $FOXP3^+$ Treg cells in the gastric cardia and antrum. In addition, the study by Silva *et al*[40], in its turn, reported that the levels of FOXP3-positive cells depend on the presence of gastritis. They observed that individuals with active chronic gastritis have lower expression of this molecule than persons without gastritis. Against this background, it is possible to infer that those cells are crucial for the occurrence of *H. pylori*-related diseases since they are directly associated with the levels of gastric mucosa inflammation.

Another finding in our study was the significantly higher levels of Treg $FOXP3^+$ cells in children than in adults in the setting of *H. pylori* infection. Along with the cytokine pattern in pediatric patients previously discussed in this paper, this data indicates a milder infection with the bacterium in infants than in older individuals. Furthermore, a recent investigation using animals observed that mice infected during the neonatal period are more intensely colonized with the bacterium than those infected during adulthood. The neonatally infected mice had an immune response characterized by an intense infiltration of $FOXP3^+$ Treg cells, and this result was VacA-dependent. Moreover, the study identified that the presence of VacA led to enhanced expression of IL-10 and TGF-β in macrophages whereas it suppressed the production of IL-23 in dendritic cells[41]. Another subsequent study by Altobelli *et al*[41] corroborates our hypothesis that the younger the host, the milder the inflammatory response against the bacterium with increased levels of $FOXP3^+$ Treg cells. They used mice to

evaluate the role of the induction of the co-inhibitory receptor B7-H1 in the chronic *H. pylori* infection and demonstrated that the induction of the Treg profile as well as the inhibition of T cell proliferation and IL-2 production are mediated by the B7-H1 expression, which results from the *H. pylori* type 4 secretion system (T4SS) action through the activation of the p38 MAPK pathway[42,43]. Interestingly, a recent study reported that animals infected with the *H. pylori* PMSS1 strain had higher levels of Treg cells and lower levels of Th17 cells than animals infected with the SS1 *H. pylori* strain[44]. Taken together, these studies show the plurality of factors influencing the induction of Treg cells in the gastric environment of *H. pylori*-positive individuals.

Notably, Wei *et al*[45] suggested that the immune response against *H. pylori* characterized by the expression of Treg FOXP3$^+$ cells and IL-10 is not only observed in the gastric mucosa, but it is also enhanced in both superior and inferior gastrointestinal tracts after 10 wk of infection, suggesting a systemic character of this regulatory immune response. Data from another study identified significant enhancement of FOXP3 expression in patients with MALT lymphoma compared to individuals with active chronic gastritis. Interestingly, *H. pylori*-positive MALT lymphoma patients with increased expression of Treg FOXP3$^+$ cells were significantly more responsive to the *H. pylori* eradication therapy than those with lower expression of Treg FOXP3$^+$ cells[46]. In addition, Sen *et al*[47] reported significant enhancement in the levels of FOXP3 expressed by T CD25+ CD127 low/- cells in the peripheral blood of patients with GC compared to the control group, and the T CD25+ CD127 low/- cells were also present in the tumor microenvironment and contributed to the suppression of T effector cells against the tumor. These results suggest a relevant role of the *H. pylori*-induced immune system regulation by FOXP3-expressing cells in the scenario of the development and progression of malignancies associated with *H. pylori* gastric infection.

Finally, we demonstrated that children infected with *H. pylori* had Treg FOXP3 cell levels positively correlated with IL-10 expression in the gastric antrum and negatively correlated with the count of mononuclear and polymorphonuclear cells. Moreover, the levels of FOXP3$^+$ Treg cells were also negatively correlated with mononuclear cells in adults. In that context, Gil *et al*[4] evaluated the expression of FOXP3, IL-10, TGF, and IL-17A as well as the dynamics of Th17/Treg FOXP3$^+$ cells in the gastric mucosa of *H. pylori*-positive children. Their data showed that FOXP3, TGF-β1, and IL-10 were remarkably expressed in the infection and the number of FOXP3$^+$ Treg cells was significantly enhanced among *H. pylori*-positive individuals compared to *H. pylori*-negatives. Moreover, FOXP3 was positively related to the bacterial density as well as with the number of polymorphonuclear and mononuclear cells among *H. pylori*-positive persons with gastritis. Therefore, the data provided by Gil *et al*[4] reinforce the influence of FOXP3 expression in the control of *H. pylori*-induced gastric inflammation and in the recruitment of mononuclear and polymorphonuclear cells, important components of the immune response against the pathogen and in the pathogenesis of diseases associated with this infection.

CONCLUSION

H. pylori infection remains an important determinant for gastric illness. Several factors can alter the host inflammation pattern directed to the bacterium, and it is evident that age is one of the most important variables in that setting. A better understanding of the immune system behavior at different ages, favoring, during childhood, the persistence of the infection and then, in adulthood, the gastric damage, can aid in the development of strategies aiming at the reduction of *H. pylori* prevalence, such as vaccines, and at the prevention of unfavorable infection-related clinical outcomes.

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REVIEW

Gut bless you: The microbiota-gut-brain axis in irritable bowel syndrome

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Abstract

Irritable bowel syndrome (IBS) is a common clinical label for medically unexplained gastrointestinal symptoms, recently described as a disturbance of the microbiota-gut-brain axis. Despite decades of research, the pathophysiology of this highly heterogeneous disorder remains elusive. However, a dramatic change in the understanding of the underlying pathophysiological mechanisms surfaced when the importance of gut microbiota protruded the scientific picture. Are we getting any closer to understanding IBS' etiology, or are we drowning in unspecific, conflicting data because we possess limited tools to unravel the cluster

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of secrets our gut microbiota is concealing? In this comprehensive review we are discussing some of the major important features of IBS and their interaction with gut microbiota, clinical microbiota-altering treatment such as the low FODMAP diet and fecal microbiota transplantation, neuroimaging and methods in microbiota analyses, and current and future challenges with big data analysis in IBS.

Key Words: Microbiota; Neurogastroenterology; Irritable bowel syndrome; Microbiota-gut-brain axis; Structural and functional magnetic resonance imaging; Machine learning; Big data analysis

Core Tip: Molecular biology, advanced neuroimaging and computer science is emerging to transform our understanding of the role of gut microbiota in irritable bowel syndrome (IBS). Herein, we provide an overview and discuss the role of gut microbiota in IBS, the clinical microbiota-altering interventions the low FODMAP diet and fecal microbiota transplantation, the role of brain-imaging and gut microbiota analyses, the importance of method selection, metadata, perspectives for improving microbiota role predictions, and big data analysis, in the seeking of understanding IBS pathology.

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INTRODUCTION

Irritable bowel syndrome – a disturbance of the microbiota-gut-brain axis

Irritable bowel syndrome (IBS) is a chronic biopsychosocial disorder manifested by recurrent abdominal pain and alterations in stool form or frequency[1]. The condition affects 4%-10% of the global population and is associated with markedly reduced quality of life[2,3]. In addition to genetic predisposition, adverse life events, psychosocial factors, chronic and acute stress, and gastrointestinal (GI) infections[1], mounting evidence suggests the gut microbiota play a key role in IBS.

Because of its heterogeneity and unclear etiology, clear biomarkers and therapeutic targets for IBS have been difficult to identify. As a term, "IBS" is collective for medically unexplained disturbances of the bidirectional communication between the gut and the brain. These disturbances are multifactorial and include visceral hypersensitivity, low-grade inflammatory responses, intestinal motility disturbances, alterations of central nervous system (CNS) processing, and alterations in gut microbiota composition[1]. In the gut, a well-functioning microbiota is highly adapted to the host and carries out biochemical and metabolic processes that are important for host function. Signals coming from the gut microbiota modulate aspects of homeostasis through neural, endocrine and immune communication pathways between the gut and the brain[4,5]. Together, this has established the concept of the microbiota-gut-brain (MGB) –axis (Figure 1).

The vagus nerve serve as a major MGB pathway modulator. It is composed of somatic and afferent fibers (80%) and general and special visceral efferent fibers (20%). Under normal circumstances, the vagus nerve sense and is activated by diet-responsive gut microbes and metabolites such as short-chain fatty acids (SCFAs), or endocrine factors, enzymes, and neurotransmitters such as serotonin, dopamine, acetylcholine, glutamate, γ-aminobutyric acid (GABA), and noradrenaline[6-9]. Each of these factors are potentially affected by alterations in microbiota composition and are involved in IBS pathology, as shown in Figure 1. In the intestines, vagal endings synapse onto neurons of the enteric nervous system (ENS), which governs the function

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of muscular, neuro-hormonal, and secretory systems of the GI tract to generate patterns of functional digestion. In IBS, the pathophysiology implicates altered gut microbiota composition, impaired intestinal mucosal integrity, and low-grade inflammation[10]. In addition to pathways through the circulatory system, several of these factors may also trigger fluctuations in the activity of the ENS with subsequent effect on the brain. This relationship of reciprocal signals may be disturbed to the degree of chronic IBS. In the chronic IBS brain, efferent signals may be perceived as unpleasant or painful, potentially leading to chronic visceral discomfort or pain[11].

The heterogeneity of the "healthy gut microbiota" has made it difficult to identify a clear IBS microbial signature. Indeed, the composition of gut microbiota composition is influenced by multiple factors, *e.g.*, geographic location, ethnicity, dietary choices, medication use, and pathogens, summarized by Adak *et al*[12]. Hence, the human gut microbiota composition is highly diverse. This heterogeneity makes is difficult to provide a clear definition to what a "healthy microbiota" is. Nevertheless, some features are considered important characteristics: a high level of diversity, a favorable amount of butyrate-producing bacteria, and resistance and resilience - the ability to withstand a disturbance promoting a shift in the composition and the attribute to return to its initial composition, functionally or taxonomical, following this disturbance[12,13]. On the contrary, in disease the microbiota composition is often associated with a decreased microbial diversity and loss of the typical balance between the host and the microorganisms[13], a so-called "dysbiosis" (a debated concept[14,15]), linked to several systemic and local human diseases.

Multiple studies have shown differences in the gut microbiota between IBS and healthy controls[16-20]. A comprehensive systematic review from 2019 showed that patients with IBS have increased levels of the bacterial families Enterobacteriaceae, Lactobacillaceae and Bacteroidales, whereas *Bifidobacterium*, *Faecalibacterium*, and Clostridiales were decreased compared to healthy controls[21]. On the contrary, Hugerth *et al*[22] recently reported no distinct microbiota signature of IBS in a random Swedish population of 3556 participants. Here, the between-sample divergence was higher in IBS compared to controls from the same population-sampling frame, but no clear biomarker of IBS was revealed. There are multiple individual reports on differences in distribution patterns of constipation predominant IBS (IBS-C), IBS with mixed constipation and diarrhea (IBS-M), and diarrhea predominant IBS (IBS-D), summarized by, among others, Liu *et al*[23], and Wang *et al*[24]. Pozuelo *et al*[25] found butyrate- and methane-producing bacteria were less abundant in IBS-D and IBS-M patients. However, Pittayanon *et al*[21] summarized six studies from 130 patients with IBS-M, demonstrating no significant difference between subtypes. Interestingly, intestinal bacterial composition has been reported to be highly dependent on sample type and regional localization. Also, mucosa-associated bacterial composition of the sigmoid colon differ between patients with IBS and healthy controls[26].

Indeed, the absence of a universal definition of what a "healthy microbiota" is, in addition to lack of consistency in sequencing methodology, study protocols, inter-individual variation that dominate intra-individual variation, definitions of "controls", and different statistical methodologies being used have made the search for a common pathological IBS microbiota signature difficult. The importance of method selection, metadata, and perspectives for improving microbiota role predictions are discussed more thoroughly in section "Intestinal microbiota analyses", below.

Another factor to consider is the impact of the circadian rhythm on gut microbiota variability. Both the level of host-derived autoantibodies and peptides and nutrient availability give fluctuations in the gut microbiota, and both are associated with circadian rhythm oscillations[6]. At least 10% of operational taxonomic units may oscillate due to the circadian rhythm, which is important to consider when collecting and analyzing fecal samples[27]. Thus, we might be in the mere beginning of understanding how alterations in gut microbiota may lead to the disruption of the intricate host-gut-microbiota-interaction. Is it a cause or a result of IBS pathology? In the last decade, much knowledge has been gained from clinical microbiota-altering interventions such as the low FODMAP-diet and fecal microbiota transplantation (FMT), which has emerged as debatably successful treatment strategies. However, their effects on the MGB-axis are still far from understood.

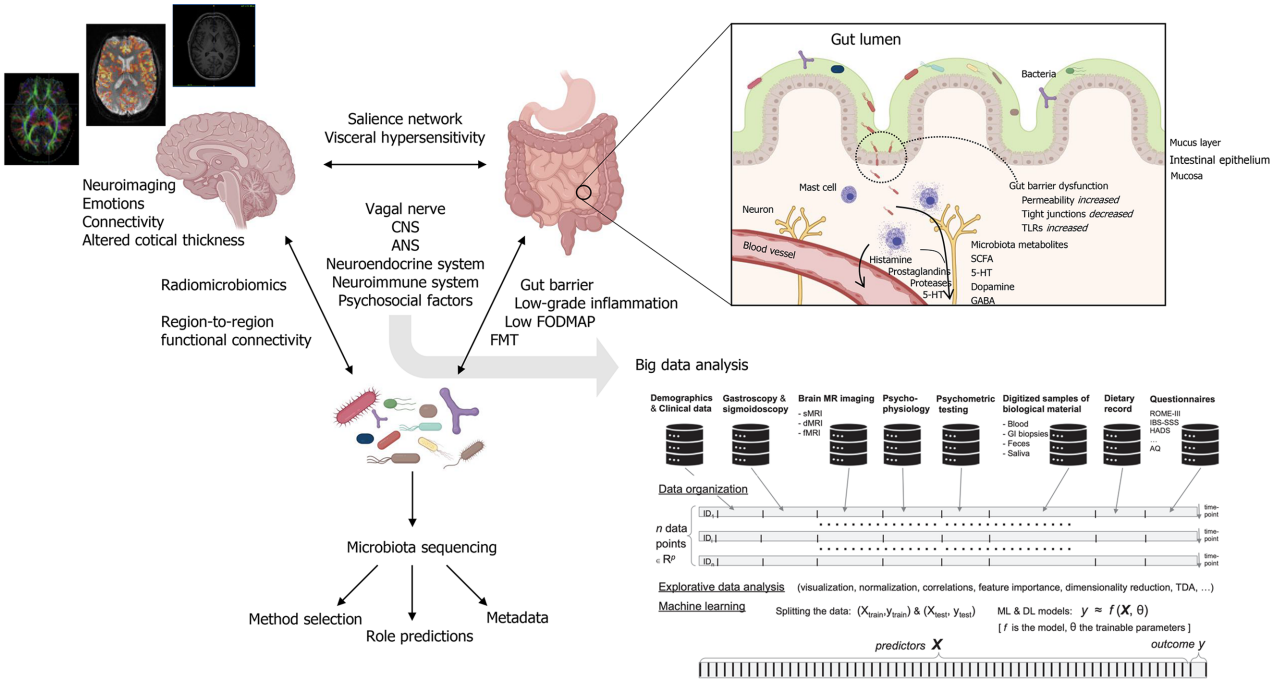

Figure 1 Integration of multimodal and interdisciplinary approaches for big data analysis in irritable bowel syndrome. Created with BioRender.com. ANS: Autonomic nervous system; CNS: Central nervous system; FODMAP: Fermentable oligosaccharides, disaccharides, monosaccharides and polyols; FMT: Fecal microbiota transplant; 5-HT: 5-hydroxytryptamine (serotonin); SCFAs: Short chain fatty acids; GABA: γ-aminobutyric acid; TLRs: Toll-like receptors.

CLINICAL MICROBIOTA-ALTERING TREATMENT IN IBS – THE LOW FODMAP DIET AND FECAL TRANSPLANTATION

Dietary intervention

Diet is an environmental factor that is pivotal in shaping the architecture of gut bacteria. Although genetics have been assumed to be of great importance[28], a recent study shows that environmental factors, such as diet, are dominating[29]. In the symbiotic host/bacteria relationship, gut bacteria depend on host intake of complex polysaccharides to facilitate growth. As hosts, humans depend on gut bacteria to break down complex nutrients resistant to human GI metabolism and metabolites produced from fermentation, such as SCFAs. In IBS, foods play an important role among the contributing factors to symptom induction. In fact, the majority of patients with IBS experience increased symptom burden after food intake[30], despite the lack of objective evidence for food hypersensitivity or allergies[31]. Several underlying mechanisms generating symptoms are proposed to be involved[32]: (1) Local effects in the small and large intestine are caused by fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs). Intake of these short-chain carbohydrates have an osmotic effect in the gut lumen, increasing small intestinal water content and introduce undigested food particles to the gut bacteria who readily ferment them, causing gas production in the colon leading to abdominal pain as a consequence of a sensitive ENS i.e.: visceral hypersensitivity[33]. In 2017, Varjú *et al*[34] compared standard dietary therapy for IBS and the low FODMAP diet in 2017. Both diets showed to alleviate symptoms, but the low FODMAP diet showed a better therapeutic effect. However, with the available data on diet interventions in IBS, a low FODMAP diet has the greatest evidence of efficacy[35], with the most updated systematic review reporting significant improvements in GI symptoms and quality of life compared to control diets or habitual diets[36]; (2) Gut microbiota alterations and bacterial fermentation might have a role in food-related symptoms. Inexplicably, contradicting findings regarding different microbiota compositions between patients with IBS and healthy controls are often reported[21,22]. A recent matched case-control study from a Thai population reported no distinction in the gut microbiota between IBS-D and healthy subjects[37]. Here, the authors accredit the discordant results from those conducted in Western countries as an effect of different dietary lifestyles affecting the gut microbiota, suggesting that alterations in gut microbiota is not the main pathogenic mechanism of IBS-D in Thai patients[37]. In the Swedish random

population, patients with IBS showed higher heterogeneity in microbiota composition compared to healthy individuals[22]. However, we need to keep in mind that bacterial fermentation capability may be more dependent on bacterial function rather than composition alone[38]. Nevertheless, differences in composition may still matter because it could result in differences in the effectiveness of a function[39]. Despite good documentation of a low FODMAP diet on symptom alleviation[36], FODMAP restriction is of concern due to possible unhealthy changes in gut microbiota composition with unknown consequences. Depriving the gut bacteria of carbohydrate and prebiotic substrates will shift the gut microbiota to ferment *e.g.*, proteins and/or some amino acids, leading to production of potentially harmful compounds, summarized by Oliphant and Allen-Vercoe[40]. Desai *et al*[41] investigated gnotobiotic mice colonized with human gut microbiota fed a fiber-deprived diet. Because of fiber deficiency, the gut microbiota fed on the colonic mucosa layer that originally acted as a defense barrier against pathogens, leading to heighted pathogen susceptibility. Multiple features of alterations in the gut microbiota composition after a low FODMAP diet are reported, such as a lower total bacteria load, a lower absolute abundance of luminal *Actinobacteria*, *Bifidobacteria*, *Clostridium cluster IV*, *Faecalibacterium prausnitzii*, and a lower concentration of the SCFA butyrate[42-44]. These studies all report on short-term interventions compared to baseline or habitual diets. The inconsistency of the study results are intriguing. McIntosh *et al*[45] comparing a low and a high FODMAP diet found a higher bacteria richness and diversity of Actinobacteria, Firmicutes, and Clostridiales in patients with IBS-D/-M in the low FODMAP group, while a high FODMAP diet decreased the relative abundance of gas-producing bacteria[45]. The sparse documentation of the long-term consequences beyond 8-12 wk of FODMAP restrictions does evoke certain skepticism. The highly restrictive nature of the diet may lead to disordered eating habits and demands much effort and motivation from patients. These factors highlight the importance of reintroduction of FODMAPs after the strict phase in the clinical management of patients with IBS[46]. Inter-individual variability and high inconsistency between clinical findings have made the search for a microbiota signature to predict treatment outcome challenging[47]; and (3) Systemic immune and inflammation responses may also contribute in symptom generation in IBS. McIntosh *et al*[45] showed that urinary histamine levels were substantially reduced following a low FODMAP diet[45], leading to hypothesizing that a low FODMAP diet might be beneficial in a subset of patients with a particular microbiota profile leading to high histamine production, hence where histamine is being a pathophysiology modulator of importance[32,45]. Our group were the first to report reduced levels of pro-inflammatory IL-6 and IL-8, but not TNF-α, after a 3-wk low FODMAP diet. Simultaneously, selected bacteria associated with anti-inflammatory properties, *e.g. Faecalibacterium prausnitzii* and Bifidobacterium, total levels of SCFAs and n-butyric acid, decreased[43]. Others have reported that SCFAs may have anti-inflammatory and immunomodulatory effects, as summarized by Tan *et al*[48], indicating that our findings present a paradox. Indeed, the full connection between diet intake, gut microbiota and its metabolites, and immune and inflammatory responses remains elusive. Herein, the intestinal barrier, gut integrity and low-grade inflammation is further discussed in section "Intestinal barrier and gut integrity", below.

Fecal microbiota transplantation

In fecal microbiota transplantation (FMT) screened stool from a healthy donor is transferred to a recipient with the purpose of altering the diversity of the gut microbiota. FMT is recommended as a therapeutic strategy in *Clostridioides difficile* (CDI) infection, and has also been demonstrated effective in inflammatory bowel disease and IBS[49]. There are multiple routes of FMT delivery available including colonoscopy, nasogastric tube, nasoduodenal tube, enema and oral capsules. Each of these modalities has been associated with varying clinical success. Additionally, whether the donor sample is fresh or frozen, or derived from a related or unrelated donor may result in different outcomes. Many recent randomized controlled trial (RCT) studies in IBS have been published, although with conflicting results. In a meta-analysis of five RCTs, overall FMT did not significantly improve IBS symptoms[50]. Here, the results were largely contradictive; one study showed amelioration of symptoms with FMT over placebo, while another study demonstrated superiority of placebo over FMT. The explanation for such contradictory results may be due to the heterogeneity of the disease. Another explanation may be the route of FMT administration. A recent double blinded RCT recruited 90 IBS patients and randomly assigned them to active treatment (n = 60) or placebo (n = 30) where fresh transplant was delivered with colonoscope to coecum[51]. FMT induced significant symptom relief in

patients with IBS, compared to controls. In 2020, our group investigated the effect of a single FMT using different stool dosages (30 g and 60 g) of frozen feces, delivered to the distal duodenum through a gastroscope. Placebo was the patient's own (autologous) feces. Here, patients responded best to the higher dosage. This study concluded that utilizing a well-defined donor with a normal dysbiosis index and a favorable specific microbial signature is important for a successful FMT[52]. Data on long-term follow-up post FMT in IBS have been sparse. However, 1-year effects were recently reported by Holvoet *et al*[53]. In a doubled blinded RCT of patients with treatment-refractory IBS with predominant bloating, patients were randomly assigned to single dose nasojejunal administration of donor stools or autologous stools[53]. Here, FMT relieved symptoms compared to placebo (autologous transplant), although the effects decreased over 1 year. A second FMT restored the response in patients with a prior response. Evidently, fecal samples from responders had higher microbiota diversity before administration of donor material than fecal samples from non-responders and distinct baseline composition, but unfortunately, no specific marker taxa were associated with response[53]. In addition, 5-year effects were recently reported in a retrospective analysis by Cui *et al*[54]. In this single-center retrospective study, patients with all subtypes of IBS were assigned to receive FMT through nasojejunal administration, colonoscopy administration, or freeze-dried capsules from healthy, screened donors[54]. Considering all patients, regardless of route of administration, 50% of patients reported gradual symptom improvement after one month to 70% and 75% after one and two years, respectively. After five years, 60% of patients experienced improvement. This decline suggests that repetitive FMT may be required for a sustained effect[54].

There are many hopes for the future in IBS treatment, and FMT capsules are one of them. Capsules are beneficial because the route of administration is much less demanding than endoscopy, they put much less stain on the patient, and can be orally administered by the patients themselves at home. Halkjær *et al*[55] performed a RCT study in patients with moderate-to-severe IBS. FMT resulted in altered gut microbiota composition, but patients in the placebo group experienced greater symptom relief compared to the treatment group after three months. Supporting this, Aroniadis *et al*[56] also found that placebo capsules did not induced symptom relief compared with placebo. Hence, the efficacy and safety of FMT in IBS is still under evaluation. Most researchers and clinicians strongly believe that more research is required before the FMT can become an openly available treatment option. A significant question that remains to be answered is whether the described dysbiosis in IBS is a consequence rather than cause of MGB-axis dysfunction in IBS. The varying abovementioned results may indicate that altering gut microbiota is not enough to obtain clinical improvement in IBS. FMT is a highly requested treatment among patients with IBS, and many practitioners find it difficult to refuse patients treatment that may be beneficial. However, researchers are calling for caution on FMT as a treatment of IBS[57]. With reference to safety, patients have reported adverse effects of abdominal pain, cramping or tenderness, diarrhea or constipation, in contrast to 2% in the placebo group[52]. In march 2020, the US Food and Drug Administration issued a warning of potential risk for serious infections due to FMT caused by enteropathogenic or Shigatoxin-producing *E. coli* that occurred following investigational use of an FMT product supplied by a stool bank, from pre-screened donors[58]. Hence, different study designs with larger cohorts are required to examine the efficacy and safety of FMT in IBS.

Indeed, the complex interplay between the host and gut microbiota is not fully elucidated, but certain features in IBS are documented to be involved, including luminal interactions and its pivotal role in the regulation of the immune system. Whether altered microbiota composition, function and abundance is the cause or a consequence of IBS, we need to understand more about their interplay with us as hosts, and importantly, the intestinal barrier and gut integrity in IBS.

INTESTINAL BARRIER AND GUT INTEGRITY

Molecular biology has revealed the presence of structural and functional alterations of the intestinal epithelial barrier and mild activation of the immune system both locally in the intestinal mucosa and systemically, in IBS. We now know that changes in intestinal permeability create a passage for microbiota and their metabolites from the lumen to the ENS, immune cells and systemic circulation, features that are associated with low-grade inflammation in IBS. Intestinal barrier dysfunction is present in a

significant proportion of reported IBS studies, especially in the IBS-D and post-infectious subtype[59]. The association between impaired barrier function and symptoms in IBS are not fully understood but visceral hypersensitivity and pain is possibly explained by exposure of the submucosal neuronal and immune apparatus. Under normal conditions, the intestinal barrier consists of a monolayer of polarised epithelial cells, coated with a thick layer of mucus[60]. As a part of the host defence system, the mucus layer entraps pathogens and is inhabited by commensal microbes such as *Bacteroides*, *Firmicutes,* and *Lactobacillus* whose products, such as IgA, contribute to the prevention of pathogen colonization[61]. Microbiota also produce proteases and protease inhibitors that can modulate the host immune response. In IBS, dysbiosis-derived proteases are thought to contribute to loss of barrier function, immune activation, and symptom generation through activation of protease-activated receptors (PARs)[62]. Recently, higher levels of fecal proteolytic activity has been identified in IBS, particularly in patients suffering from post-infectious IBS[63]. Notably, this was associated with changes in microbiota composition, suggesting that specific microbes contribute to increased production or inadequate suppression of proteases and subsequent activation of PARs that may lead to intestinal barrier dysfunction.

In normal barrier permeability, the space between epithelial cells are sealed by tight junctions and maintained by a complex network of protein interactions. In IBS, the expression levels of tight junction proteins, such as Occludin, Zonula occludens-1, and Claudin-1, have been found to be reduced in the duodenum, jejunum and colon[59,64]. Interestingly, microbiota has been found to regulate the expression of tight junction proteins[65], and enhanced bacterial passage over the barrier has been observed[66].

In the intestinal mucosa, mast cells comprise 2%-3% of the immune cell pool of the lamina propria, and increased intestinal mast cell concentration or activation is one of the most consistent pathological findings in IBS[67-69]. Mast cells possess a great number of stimulatory molecules which allow interaction with a multitude of partners, including immune and non-immune cells. Activated mast cells release mediators such as histamine, serotonin, proteases, and prostaglandins, and they also secrete cytokines and chemokines. Their interactions are indeed complex. Some mast cells interact with both the commensal microbiota and the nervous system by signalling to enteric neurons though serotonin while being influenced by neurotransmitters such as substance P or noradrenalin[70]. In IBS, mast cell-induced activation of enteric neurons may contribute to visceral hypersensitivity[66,71]. Indeed, some bacteria specifically affect mast cells function and activation, but the role of dysbiosis-mast cell-interaction in IBS is yet to be elucidated.

A potential marker of low-grade inflammation in IBS is altered levels of cytokines. Multiple cytokine profiles of patients with IBS have been reported, but they are highly inconsistent. Some has found increased levels of circulating pro-inflammatory cytokines such as IL-6, IL-8, IL-17, and TNF-α[72-74], or reduced levels of the anti-inflammatory cytokine IL-10[72]. Other studies indicate no difference between patients with IBS and healthy controls [75,76]. Interestingly, associations between altered cytokine profiles and changes in gut microbiota have been observed. A study by Hustoft *et al*[43] found reduced levels of IL-6 and IL-8 and decreased levels of Actinobacteria, Bifidobacterium, and *Faecalibacterium prausnitzii* after three weeks of the low FODMAP diet. Changes in cytokine levels could thus indicate an abnormal mucosal immune response associated with changes in the gut microbiota.

Increased expression of Toll-like receptors (TLRs) is an interesting finding in patients with IBS[77,78]. These receptors are found on many different cells, including intestinal epithelial cells and immune cells, and they interact in close relation to neural and immune receptors that are involved in the homeostatic regulation in the gut mucosa[79]. TLRs recognise specific microbial components of both commensal and pathogenic bacteria and play a role in immunologic tolerance to commensals and defend against pathogens[80]. In association with increased levels of TLRs, changes in both cytokine profiles and gut microbiota have been observed in patients with IBS, suggesting that an altered microbiota profile may influence TLR expression and immune activation[78].

Indeed, dysbiosis may induce intestinal barrier loss and increased intestinal permeability that cause bacterial products and metabolites to permeate the epithelial barrier, thus triggering an inflammatory response[81]. Mast cells are ''gate keepers'', and they are not only involved in allergic reactions, but also in host defence including recruitment and activation of other immune cells which may evoke the symptom generation. We believe that further studies should be more focused on which triggering factors that are involved in the link between gut microbiota, intestinal permeability, and intestinal mucosal response in patients with IBS. Indeed, changes in

intestinal permeability create a passage for microbiota and their metabolites from the lumen to the ENS, immune cells, and systemic circulation, features whose effect on the brain should also be investigated.

NEUROIMAGNING AND GUT MICROBIOTA IN IBS

The MGB-axis represent a paradigm shift in both neuroscience, gastroenterology and systems medicine. See Mayer *et al*[82] for a visionary and integrative systems-biology-based model approach to IBS. GI symptoms such as heartburn, indigestion, acid reflux, bloating, pain, constipation, and diarrhea can be triggered by emotional and psychosocial factors. Conversely, GI symptoms alter CNS processing in the absence of detectable organic disease and are implicated in neurological disorders and psychiatric conditions such as anxiety, depression, autism spectrum disorder (ASD), and Parkinson's disease. Brain imaging modalities and techniques are valuable tools that can non-invasively extract both structure and function in the living brain at the millimeter scale and at a temporal resolution down to seconds. To study the IBS brain, or more generally, applying brain imaging to explore disorders of gut-brain interaction and relation to gut microbiota, the most important modality with whole brain coverage is likely magnetic resonance imaging (MRI)[83,84]. Among the plethora of MRI measurement techniques, there are (1) Structural MRI (sMRI), providing 3D images with high spatial resolution and various types of soft tissue contrast enabling quantitative assessment of brain morphometry such as volumes of different brain structures or regions, local and patch-wise cortical thickness and gyrification, and localized MR signal intensity patterns, *e.g.*, radiomics[85]; (2) diffusion MRI (dMRI), measuring directional and tissue-dependent water diffusion at the microscopic scale enabling quantitative assessment of tissue microarchitecture, *e.g.*, metrics such as fractional anisotropy (FA) derived from the voxel-wise diffusion tensor estimation, and large-scale structural connectivity between brain regions obtain by fiber-tracking algorithms; and (3) functional MRI (fMRI), based on blood-oxygen-level-dependent (BOLD) contrast imaging sensitive to local neuronal activity across the brain in situations where the brain is exposed to cognitive, emotional or sensory stimuli given under experimental control, or being in "resting state" where brain activity is assumed to be intrinsic due to spontaneous fluctuations in the paramagnetic BOLD signal and thereby detectable even in the absence of an externally prompted task or a specific sensory stimulus. A large proportion of neuroimaging studies, partly also targeting IBS, have focused on structural and functional brain connectivity using a combination of dMRI and/or fMRI recordings and topological network analysis based on graph theory[86-89], and more recently also deep learning methods and graph convolution approaches for functional annotation of cognitive states[90,91]. It is expected that these deep learning methodologies will also penetrate imaging and network-based analysis in IBS research[82].

Keeping the systems view on IBS, the term *radiomicrobiomics* was coined by De Santis, Moratal and Canals in their perspective paper on advancing along the gut-brain axis through big data analysis for diagnostic and prognostic purposes[92]. The term was introduced with reference to the efforts of combining microbiota sequencing data from the gut microbiota with imaging-based features that can be obtained from the conversion of brain images into mineable tabular data or graph representations in a network context. Interestingly, the gut microbiota seems to influence complex physiological systems other than the gut-brain axis and the pathophysiology of IBS, systems that are homeostatically regulated, partly involving CNS and ANS processing. These are blood pressure and the development and pathogenesis of hypertension, glycemic control, development of obesity and diabetes, mood regulation, and anxiety and depression[93-96]. In all these cases, neuroimaging and network analysis will be an important window to the brain and its interplay with microbiota composition and dynamics. More specifically, exploring the associations between neuroimaging parameters, such as brain regional volumes and gray matter densities assessed with sMRI, microstructural patterns assessed with dMRI and derived FA-values, or interregional functional connectivity in *e.g.* the salience network, visceroceptive, pain processing, or emotion-regulating networks assessed with resting state fMRI and specific gut microbiota signatures, has the potential of vastly enhancing our knowledge on gut-brain interactions in IBS. In neuroimaging of the IBS brain, one of the most consistent findings are alterations in the structure and function of key regions of the somatosensory network, including the globus pallidus, putamen, and caudate, composing the basal ganglia[83]. It has also been reported increased gray matter

density (GMD) in the hypothalamus and decreased GMD in the prefrontal cortex in the IBS brain[97]. In rectal distention experiments, patients with IBS had a differential brain response in the pain matrix and default mode network[89]. Neuroimaging studies has also revealed gender-differences in IBS brain network alterations. Female IBS patients showed increased cortical thickness in the pre- and post-central gyrus and decreased thickness in the bilateral insula and the left subgenual anterior cingulate cortex (sgACC), compared to healthy female controls. Connecting emotions and altered brain function in IBS, patients with IBS and comorbid alexithymia have different brain responses to rectal distention in the right insula[98]. As a first in our field, Norlin *et al*[99] recently provided evidence that the vulnerability to fatigue in IBS is associated with connectivity within a mesocorticolimbic network as well as immune activation in the form of enhanced plasma levels of TNF-α, compared to controls. Indeed, there has been published a large series of papers on brain imaging in IBS, and now, evidence for disrupted subcortical and cortical regions mediated by gut microbial modulation are emerging. Labus *et al*[100] reporting associations between brain region-to-region functional connectivity and microbiota found a correlation between Clostridia and Bacteroidia with connectivity of the thalamus, the basal ganglia (caudate nucleus, putamen, pallidum, nucleus accumbens), the superior part of the precentral gyrus, the anterior insula and ventral prefrontal regions. Recently, the same lab also reported on fecal metabolites and resting state fMRI[101]. Here, the differences in histidine, cysteine, glycine, glutamate, spermidine, and anserine were significantly associated with the alteration in left dorsal part of the posterior cingulate gyrus to the left putamen. Also, the changes in histidine, tryptophan, uracil, 2-deoxyuridine, thymidine, and succinate were differentially associated with the alteration in the right superior frontal gyrus to the right putamen. Interestingly, this interaction may be mediated by aberrant tryptophan signaling in IBS, which is important because it is a substrate for serotonin synthesis.

In combining brain imaging data, molecular and genetic data, and metagenomic data for joint analysis, new challenges and opportunities arise in the attempt to elucidate the mechanisms and biomarkers of IBS. This endeavour is further described and discussed in the section "Big data analysis" below.

INTESTINAL MICROBIOTA ANALYSIS

Importance of method selection

Microbiome research is advancing rapidly, improving the precision of taxonomy and functional surveys and minimizing methodological limitations. After almost two decades of the earliest intestinal microbiota surveys in humans, we have advanced towards recommendation of quasi-standard methodological procedures to make next-generation sequencing (NGS) data comparable across studies. Notwithstanding, the complete implementation of standards is challenging, given the wide variety of commercial options for sampling, DNA extraction, amplicon generation, library preparation, and DNA sequencing that users fit at their own convenience, making cost-effectivity prevailing. In this regard, the International Human Microbiome Standards consortium has agreed that stool sampling requires minimal processing (no addition of preservation buffers and nuclease inhibitors) to maintain microbiota DNA and RNA integrity, thus facilitating sampling, storage and transport logistics by donors and patients. Besides, stool sub-sampling was revealed to produce minimal variation within individuals[102,103]. Among the multiple methodological steps, DNA extraction introduces the larger variation across experiments, and chemical (muralytic enzymes) or physical cell-wall disruption (bead-beating) methods during DNA extraction are recommended to gain the representation of some microbial species during massive and parallel sequencing and taxonomy data appraisals[103,104]. Taxonomy surveys *via* amplification and sequencing of bacterial 16S rRNA gene are widespread because of their cost-effectivity. A large collection of tools (*e.g.*, QIIME2, Mothur, DADA2, *etc.*) and reference repositories (SILVA, RDP, Greengenes, GTDB) have been developed for such an aim. However, this methodology allows reliable identifications mostly at the family and genus levels. Also taxonomy classification depends on the region amplified[105,106] and, accordingly, inconsistencies have been found across studies. Currently, most of the studies are sequencing the V4 or V3-V4 hypervariable regions because of their larger genetic variation and discriminatory power, facilitating re-use and comparisons across different studies.

Metagenomic analysis, based on non-targeted massive DNA sequencing, outperforms the 16S rRNA gene hypervariable region sequencing, although it is much more expensive. Gigabase-level information normally recovered from individual samples permit inspection of microbial species present in human samples and other ecologically complex environments[107,108]. The discriminatory capability of this approach is constantly improved thanks to the existence of comprehensive genome catalogues[109], compiling a huge amount of microbial genetic variability and making it possible sample profiling at the strain level[110]. In addition to the detailed taxonomy surveys, metagenomics makes functional appraisals feasible *via* DNA read mapping strategies using curated and comprehensive repositories (*e.g.*, KEGG, COG, eggnog databases). The functional analysis has also been developed for 16S rRNA gene amplicon sequencing data (namely PICRUSt). However, the predictions made with such an approach have a high degree of uncertainty due to the ambiguous taxonomic assignments of the 16S rRNA readouts and absence of functional variation information at the species level (minimal gene/functions shared on multiple genome examinations – pangenome – within a single species).

Metadata does matter

The interpretation of microbiome data require a proper control of covariates that many times are not available to be incorporated into the data analysis. This could lead to ambiguous (relying on the generally recognized strain-specific pathogenicity traits) and uncertain (plenty of false-positives) associations between the microbiota and health and disease states, largely influenced by confounding variables[111-113]. Of environmental factors, the intestinal microbiota is strongly influenced by the dietary patterns. Therefore, the use of dietary records around the sampling time are good strategies to integrate such information in the data analysis. The value of this type of information is even more important in microbiota-based biomarker discovery for example for IBS given the impact of food intake on disease symptoms[114]. Nevertheless, not all studies have found meaningful differences in microbiota when using dietary records[115,116]. There is growing evidence supporting a role of energy and macronutrient intake on the intestinal microbiota which could affect associations with health and disease. Body mass index (BMI), gender and age could affect both dietary habits and the intestinal microbiota and change through life differently in women and men[117-119]. By integrating gender, BMI, diet, and age information with microbiota data, the results are less influenced by the subjects' idiosyncratic variation and signals looking for links between gut microbes and health/disease states become more reliable. Moreover, pharmacological treatments given to IBS patients to tackle symptoms should be considered as they could bias the conclusions on the microbiota signatures correlated and playing a role in IBS. There is indeed extensive impact of non-antibiotic drugs on the composition and metabolic function of the gut microbiota [14,120]. In summary, good practices in microbiome research for clinical application undoubtedly involve a meticulous metadata recording covering a large set of individual and lifestyle information that permit uncover unquestionably the influence of gut microbes in our health.

Perspectives for improving microbiome role predictions

The integration of functional omics provides information on the potential role of intestinal microbes and the metabolic products resulting from host-diet-microbe interactions and allows generating human-data-driven hypotheses which could be latter validated in study models. These methods turn data processing more complex because of multidimensionality, but provide clues on the molecular mechanism driven by microbe-host interactions and underlying health and disease[121]. For instance, the correlations of gut microbes (metagenomics) with secondary bile acids (lipidomics), SCFAs (metabolomics), and pro-inflammatory molecules (proteomics/cytokine arrays) make it possible to distinguish microbial groups that plausibly explain disease states or the physiological response to a particular dietary components[122-124]. The high individual specificity and variability of the microbiome data also requires the application of statistical methods that minimize false-positives during biomarker discovery, permit an adequate covariate control and integrate other multidimensional datasets. In this context, the EU COST action ML4Microbiome represents an interesting initiative for advising, researching, and developing advanced statistical and machine learning approaches applied to microbiome research that could greatly contribute to standardizing and improving data analysis in this field[125,126].

Promising developments have also emerged to improve, for example, the accuracy of the dietary assessments. These are often based on self-reported data and, therefore, biased affecting the interpretation of its relationship with the microbiome and health

outcomes. In this regard, metabarcoding of plant DNA has been proposed as a method to tacking human plant intake more accurately than using dietary questionnaires. Although this strategy has been only applied to gain information on plant components of the human diet, it looks promising to infer the dietary intakes and the resulting diet-microbe interactions[127].

Sequencing technological advances are also helping to improve the taxonomy resolution of targeted amplicon-based microbiota analysis. The emergence of single-molecule sequencing platforms (Oxford Nanopore Technologies and PacBio) has permitted to generate longer DNA reads, pivotal to increase the information of gene markers under inspection in microbial diversity assessments and to gain resolution at the species-level at a lower cost than metagenomics[128,129]. This methodology has already demonstrated good performance on microbiota surveys despite the modest per-base quality of its reads compared to the classical Sequencing-by-Synthesis (SBS)-based instruments (Illumina). Its potential is even more promising to infer strain-level variation pivotal to determine, for example, species engraftment after FMT[130], which, as mentioned above, is under investigation to treat IBS[52,131].

BIG DATA ANALYSIS

The enormous potential of big data, when harnessed efficiently by powerful statistical methods, mathematical models, and machine learning algorithms, often translates into deeper insights in multi-factorial dynamic systems, which are otherwise complicated to explore, describe and comprehend. The MGB-axis is a well-suited example of such a complex multivariate system and data science *(i.e.*, machine learning algorithms and statistical analyses) is the method of choice to approach this problem. It has become highly evident that no single factor underlie the heterogeneous disorder of IBS. Its investigation requires analysis of large datasets collected from an array of clinical disciplines for a deeper understanding of pathophysiological mechanisms and pathways, and correlations with specific symptoms and symptom severity.

The large number of factors involved both from the brain and microbiota along with their continuous variability, yield large amount of information. These factors are probed using various clinical modalities across several points in time in longitudinal research. The number of resulting variables can range from few hundreds to tens of thousands and the magnitude of data can easily approach hundreds of thousands to several millions of data points, even for studies recruiting several tens of participants. This sheer scale of data combined with larger dimensionality and significant variability sets up a ripe case for employing sophisticated data science methods to study intricate relationships between brain and gut microbiota.

The MGB scientific community appear to already recognize and acknowledge the importance of data science in the field of our research. For example, in their review, De Santis *et al*[92] described the potential of large amount of information emerging from advanced neuroimaging systems and sophisticated microbiome sequencing techniques to probe complex interactions in the MGB-axis. Their proposal was to combine data from both of these domains to analyze quantitative features for intricate relationships using computational analysis methods, a process they termed “radiomicrobiomics”, which could potentially unveil novel biological information on the MGB-axis. Similar ideas were expressed in another critical review whose key note suggested that encoding microbial information along with other necessary variables into machine learning algorithms could excel our understanding on GI disorders, which are challenging to diagnose due to multifactorial nature of underlying pathology[132]. Mayer *et al*[83,133] had stressed the need of integrating large sets of host's multi-omics data and microbial data with machine learning techniques to reveal novel insights into the MGB-axis, independent of existing theories and hypothesis. Kaur *et al*[134] also emphasized the role of machine learning in multiomics data analysis to probe MGB relationship and discussed a framework to move beyond prediction to prevention and personalized therapeutics in MGB related disorders.

In line with these proposals, reports of initiatives and on-going work where the MGB-axis is being explored using the discipline of data science are beginning to emerge. In a recent study, statistical analyses were performed on combined brain and microbial datasets, acquired using resting state fMRI and genetic sequencing, respectively[135]. Probably for the first time, clear correlation-based associations were drawn between certain species of microbiota and corresponding brain regions affected by it, a step forward in the right direction. Wu *et al*[136] studied the association of gut microbiome in ASD using an array of statistical and machine learning-based analyses

and realized presence of certain bacterial genera in ASD group, which could be used as a potential ASD biomarker. Stevens *et al*[137] studied association of depression phenotype with gut microbiome using microbial genetic information at single nucleotide resolution using multivariate analysis. Based on genetic data of microbiome, they were able to differentiate between depression and healthy cases. The Bergen brain-gut-microbiota study is a notable example of an on-going work that integrates data science with multiomics, where both brain and gut data is being collected from an IBS patient cohort and healthy controls, as shown in Figure 1[138]. BrainGutAnalytics, an advanced analysis project under the umbrella of the Bergen brain gut study, aims to apply sophisticated data science methods to locate IBS biomarkers in brain and peripheral organ systems[139].

Despite these rapidly growing applications of data science in investigating the MGB-axis, it appears that the full potential of data science is yet to be leveraged. Data science techniques, such as machine learning models, particularly thrive in scenarios where the sample size is high (*i.e.*, in order of thousands or more), as it allows the models to adequately learn the underlying data structure by iterating over large number of observations. On the contrary, clinical studies are often limited by sample size albeit high dimensionality of data, as various studies report participant cohorts comprising a few tens to a few hundred subjects only[120-122]. This limited sample size, on one hand, impedes the development of reliable computer models and on the other, high feature to sample ratio could lead to overfitting of the model, which often result in misleading predictions[119]. One tangible way to address this problem is aggregation of several datasets coming from various small scale studies into a larger MGB-axis database, as also proposed by other researchers[80,118,121]. Such a collection will not only feed the needs of data starving computer models but will also represent diverse sectors of subject population in terms of demographics and genetic backgrounds, improving generalization and validation of analysis outcomes. However, such an initiative would only be meaningful if a highly controlled and uniform system of data collection could be developed and implemented across all participating studies. A merger of various datasets taken from isolated studies following their own highly customized protocols, based on variable inclusion and exclusion criteria, will not carry much scientific value. Similarly, an acceptable level of consistency in data management system across all studies is imperative for rapid data accessibility, interpretation and interoperability. The establishment of a larger MGB database would also facilitate much needed interdisciplinary collaboration in MGB research, as data scientists and clinicians must join forces together to solve this complex puzzle.

CONCLUSION

In recent years, research aiming to understand the influence of the gut microbiota on bidirectional interactions between the gut and brain has gained momentum. As described and discussed in this review, the role of microbiota in IBS is so multifaceted that it requires research approaches across disciplines and scientific fields, to reveal details of the complex interactions. Currently, most dietary or FMT interventions are limited to observations of transient microbial shifts within short time frames. Personalized responses of the host microbiota may explain some of the heterogeneity of research outcomes, but not all. Because IBS fluctuates between periods of remission and aggravation of symptoms, longitudinal sampling, multiple sample time-points, post-intervention follow-ups and washout periods for cross-over studies are needed to identify microbial changes that are missed when using cross-sectional sampling, will be of great importance in future studies. We know that gut microbiota profiles are significantly associated with alterations in intestinal gut integrity, brain microstructure, intrinsic neural activities, and cognitive function and mood. How this tremendously intricate symbiotic relationship works in IBS, remains to be unraveled. Multimodal and interdisciplinary clinical studies that include assessments of the gut microbiota composition and function in conjunction with neuroimaging and behavioral testing, such as the Bergen BrainGut microbiota-study[138] are necessary for verification of directionality and causality in the MGB-axis in IBS. Other important work to come are how probiotics influence gut microbiota and affect functional changes in the brain through gut microbiota[140]. As described in this review, the choice of method for analysis is important. We believe, that only through integration of multiple advanced techniques, such as metabolomics and neuroimaging, can we generate a complete picture of host and microbiota pathways in IBS.

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REVIEW

Sarcopenia in hepatocellular carcinoma: Current knowledge and future directions

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Abstract

Liver cancer is the second most occurring cancer worldwide and is one of the leading causes of cancer-related deaths. Hepatocellular carcinoma (HCC) is the most common (80%-90%) type among malignant liver cancers. Sarcopenia occurs very early in HCC and can predict and provide an opportunity to improve muscle health before engaging in the treatment options such as loco-regional, systemic, and transplant management. Multiple prognostic stating systems have been developed in HCC, such as Barcelona Clinic Liver Cancer, Child-Pugh score and Albumin-Bilirubin grade. However, the evaluation of patients' performance status is a major limitation of these scoring systems. In this review, we aim to summarize the current knowledge and recent advances about the role of sarcopenia in cirrhosis in general, while focusing specifically on HCC. Additionally, the role of sarcopenia in predicting clinical outcomes and prognostication in HCC patients

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Grade D (Fair): 0
Grade E (Poor): 0

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undergoing loco-regional therapies, liver resection, liver transplantation and systematic therapy has been discussed. A literature review was performed using databases PubMed/MEDLINE, EMBASE, Cochrane, Web of Science, and CINAHL on April 1, 2021, to identify published reports on sarcopenia in HCC. Sarcopenia can independently predict HCC-related mortality especially in patients undergoing treatments such as loco-regional, surgical liver transplantation and systemic therapies. Basic research is focused on evaluating a balance of anabolic and catabolic pathways responsible for muscle health. Early clinical studies have shown promising results in methods to improve sarcopenia in HCC which can potentially increase prognosis in these patients. As sarcopenia occurs very early in HCC, it can predict and provide an opportunity to improve muscle health before engaging in the treatment options such as loco-regional, systemic, and transplant management. Further, sarcopenia measurement can obviate the confounding caused by the abdominal ascites in these patients. The use of sarcopenia can add to the existing scoring systems to better prognosticate the HCC.

Key Words: Sarcopenia; Skeletal muscle; Hepatocellular carcinoma; Cirrhosis; Outcomes; Liver cancer

Core Tip: Sarcopenia is a condition defined by the loss of skeletal muscle mass, quality and strength. It is commonly seen as a part of normal aging but can also be noted in multiple conditions such as chronic inflammation, cancers and use of drugs. Sarcopenia is common in liver cirrhosis and is associated with overall poor outcomes (disease-free survival). Recently, the adverse effects of sarcopenia in hepatocellular carcinoma (HCC) has been an area of intense interest. Altered bio-impedence and rapid muscle loss in liver diseases could alter skeletal muscle strength in these patients. Additionally, development of tumor-related cytokines can accelerate the sarcopenia progression which could provide insights into disease progression and response to various therapeutic options. While multiple scoring systems are available to evaluate the HCC progression, sarcopenia provides an additional functional status tool to further refine these systems. In this article, we summarize the role of sarcopenia in HCC progression and changes during locoregional and systemic treatments.

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INTRODUCTION

Primary liver cancers include hepatocellular carcinoma (HCC) and other non-HCC tumors. Primary liver cancers are the second most lethal cancer worldwide, fourth leading cause of cancer mortality and sixth frequently diagnosed cancer per year[1]. HCC is the most common cancers among the primary liver cancers, which constitutes 90% of cases. HCC usually develops within a liver cirrhosis (cirrhotic-HCC, 80% of cases), and rarely with no appreciable cirrhosis or advance fibrosis (non-cirrhotic-HCC, 20% of cases)[2]. Due to aggressive nature of HCC, prognosis is poor. This is compounded by delay in the treatment, limiting life expectancy and management options. Early identification of high-risk features for appropriate stratification, and prognostication in HCC is paramount to alter the disease course and improve survival. Several prognostic staging systems and biomarkers have been developed to identify the patients at risk of poor prognosis[3]. Some of these include Cancer of the Liver Italian Program, Barcelona Clinic Liver Cancer (BCLC), Child-Pugh score, Chinese University Prognostic Index score, the Hong-Kong Liver Cancer stating system and

Japan Integrated Staging. Further, biomarkers such as alpha-fetoprotein (AFP), des-γ-carboxyprothrombin AFP-L3, vascular endothelial growth factor, and angiopoietin 2 were used as independent prognostic factors in advanced tumors[4]. However, current available staging and prognosticative systems lack parameters that consider nutritional, functional and performance status[5]. Although long-term prognosis is dependent on the liver reserve and staging of the cancer, poor performance can significantly affect clinical outcomes in HCC patients. The use of the Eastern Cooperative Oncology Group classification with BCLC could provide an assessment of patients functional status.

Rosenberg[6] introduced the term "Sarcopenia," which was coined from the Greek word "sarx," or "flesh," and "penia," or "loss." It can be defined as loss of skeletal muscle mass, quality, strength with a reduction in the motor unit number, atrophy of type muscle fibers[7], and can contribute to frailty, functional impairment, and disability[8-11]. Three most commonly used diagnostic criteria used for sarcopenia include "muscle mass" (height-adjusted), "muscle strength," and/or "physical performance"[12]. A focus on muscle function has shown to be a powerful predictor of clinically relevant outcomes rather than muscle mass alone[13]. Recently, body mass index (BMI)-adjusted mass is found to be a better predictor of adverse outcomes than height-adjusted muscle mass[14,15]. Further, multiple muscles or groups of muscles could be utilized to assess sarcopenia. Some of the most commonly used muscles include the paraspinal muscle area (psoas muscle, quadratus lumborum, transverse spinal muscle, erector spinae muscles) and triceps muscles (mid-arm circumference). Loss of skeletal muscle mass can affect static, dynamic and isokinetic strength[16]. It can also be associated with a decline in the maximum oxygen consumption (at a rate of 3%-8% per decade of life starting from 30 years) which ultimately leads to a decrease in overall functioning[17]. Dynamic changes in skeletal muscle mass and function can occur with changes in hormones (daily insulin, glucagon), anabolic steroids, corticosteroids, thyroid (month-to-month), and immune mediators [interleukin (IL)-1, tumor necrosis factor, and IL-2]. Primary sarcopenia is noted to be due to physiological states such as aging and secondary causes (acute or chronic illness)[18]. Individuals with cancer may deplete up to 80% of their muscle mass. Further, sarcopenia can be noted in as high as 80% and 60% of patients with upper gastrointestinal and lung cancers, respectively[19]. Pre-therapeutic sarcopenia is noted with highest prevalence in esophageal and small-cell lung cancers and could have severe consequences in terms of post-operative complications, chemotherapy-related toxicity, and poor overall survival (OS)[20].

Cross-sectional imaging is commonly performed in HCC patients for diagnosis, surveillance, and treatment response[19]. It is logical to use this cross-sectional imaging to evaluate skeletal muscle mass simultaneously for valuable information to assess the prognosis and treatment outcomes. Additionally, patients with cirrhosis and HCC commonly develop ascites spuriously increasing the abdominal girth and weight. Despite this increase, significant proportion of these patients have decreased muscle mass leading to "sarcopenic obesity[21]." Use of an objective tool (which is measurable and reproducible) to assess the survival of HCC patients with ascites remains a challenge. Furthermore, methods to assess the prognosis of HCC patients during/after loco-regional (radiofrequency ablation, radioembolization, chemoembolization), liver transplantation, and systemic therapy (chemotherapy, immunotherapy) could have a long-lasting impact on these individuals. One such objective method is to use sarcopenia to assess the patient response and overall could assist in OS in HCC patients[14,22-34]. Therefore, this manuscript aims to describe the role of sarcopenia in the management and prognosis in HCC. Furthermore, we aim to describe and summarize the methods to improve sarcopenia to enhance the survival of patients undergoing treatment for HCC.

LITERATURE SEARCH

An electronic search was performed using databases PubMed/MEDLINE, EMBASE, Cochrane, Web of Science, and CINAHL on April 1, 2021, to identify published reports on sarcopenia in HCC. We used the following search terms- "carcinoma, hepatocellular" or "cancer, hepatocellular" and "sarcopenia" or "sarcopenias". A total of 4762 articles were published on sarcopenia and 167571 on hepatocellular cancers. Both basic science and clinical studies were included. A combined search revealed 2289 articles over the last 12 mo. The authors AP and HG reviewed the articles independently. Clinical reviews, case reports, and case series were excluded. A manual search was

performed by evaluating the references from included studies and related articles in multiple databases. If any discrepancies, these articles were re-reviewed by the author RT. After removing non-relevant/duplicates/non-English language articles, including a manual search, 80 full length published articles were finally reviewed.

HCC AND SARCOPENIA

Secondary sarcopenia is a common finding in patients with cirrhosis. Reduction in protein synthesis can lead to a decrease in lean body mass seen in cirrhotics[26]. Protein catabolism seen in disease processes such as neoplasms can lead to significant loss of muscle mass and it can be seen up to 40% of patients with liver cirrhosis[35]. Sarcopenia can be associated with an increased risk of encephalopathy, post-transplant mortality, infections, treatment effectiveness, and quality of life[36-38]. Patients with cirrhosis who were diagnosis with HCC showed accelerated sarcopenia up to 30-40% at the time of diagnosis[39,40]. Sarcopenia in these patients can independently predict HCC-related mortality along with decompensated cirrhosis, performance status, TNM staging, and BCLC class[41,42]. However, each of these have limitations with biggest being lack of prognostication, inability to provide comprehensive tool to assess complex interactions between cirrhosis, HCC and functional capacity[43]. Further, factors responsible for survival differ significantly among patients with compensated and decompensated cirrhosis[44].

As HCC occurs over time in patients with underlying chronic liver disease, assessment of skeletal muscle mass and change overtime can provide important details about deterioration of the disease. A number of tumor-related factors (cytokines and myokines) can change the skeletal muscle mass which can assist to further refine these scoring systems. Furthermore, cirrhotic have ascites, disproportionate loss of muscle compared to fat (altering BMI) leading to difficulty in interpreting bioimpedance, anthropometric measurements. Hence use of tools to integrate degree of sarcopenia-related measurements by CT-based techniques can offer ways to predict change in these patients[45,46].

BIOLOGICAL BASIS OF SARCOPENIA IN HCC

Sarcopenia is the condition characterized by loss of muscle strength, mass, and functional ability. The pathophysiology of this muscle loss can be multifactorial (hormonal, inflammatory, age-related, chronic liver and non-liver states, drug induced). Loss of muscle anabolic activity with nutritional deficiency can further worsen sarcopenia. Loss of skeletal muscle homeostasis especially between hypertrophy and regeneration can lead to sarcopenia. Most of the changes related to sarcopenia originates with normal aging process. A balance of muscle protein anabolic and catabolic pathways are responsible for muscle health. During sarcopenia, multiple cellular changes occur such as the reduction in myofibres (size and number), myosteatosis (development of intramuscular and intermuscular fat infiltration)[47], decreased number of type II fibre satellite cells. Further, loss of mitochondrial integrity, molecular signaling [IGF-1, mammalian target of rapamycin complex 1 (mTOR)], neurological (plaque formation, motor neuron loss), epigenetic change (modulated *via* microRNAs), endocrine factors (myostatin , osteocalcin and abnormal communication among them) and reactive oxygen species (ROS) imbalance[48] combined with reduced physical activity can all contribute to the muscle loss. Some of the frequent causes of sarcopenia are elucidated in Figure 1. Hyperammonemia, increased autophagy, decreased protein synthesis, abnormal mitochondrial activity, increased proteasomal activity, and low testosterone levels are also responsible for sarcopenia cirrhosis[49,50]. It is compounded by decreased metabolic substrates (especially branched-chain amino acids)[51], extrahepatic gluconeogenesis, and increased insulin resistance/pro-inflammatory cytokines (NFκB signaling, mTOR inhibition, enhanced apoptosis, eukaryotic initiation factor-2[52]. Portal hypertension-related complications and alcohol intake further worsen sarcopenia in cirrhosis[53,54].

Early sarcopenia can be seen in HCC individuals[24,55]. Myokines (myostatin, IL-6, follistatin) are cytokines produced and secreted by muscle fibers and can exert paracrine/autocrine effects[33]. Myokines can exert immunological and anti-inflammatory effects and facilitate proinflammatory state of liver fibrosis, cirrhosis, and HCC development. Although myostatin levels in HCC have been a matter of debate, high IL-6 and follistatin levels had a significantly lower 5-year OS rate in HCC and were

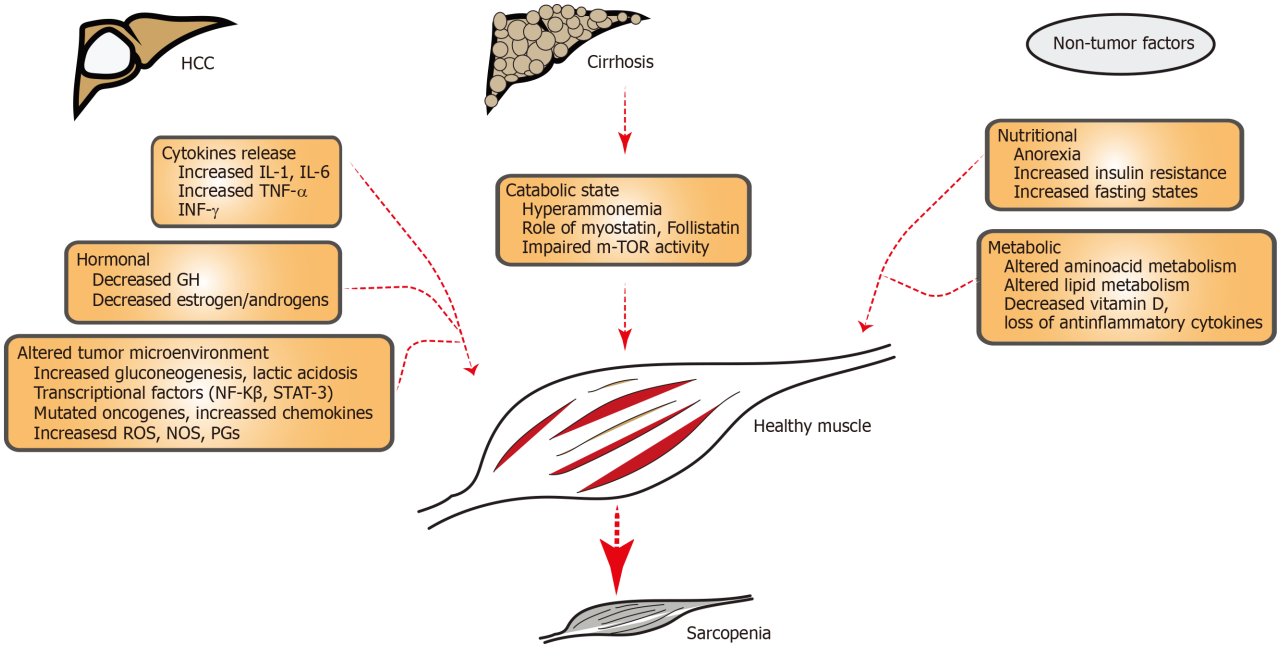

Figure 1 Schematic illustration showing factors contributing to sarcopenia in hepatocellular carcinoma and cirrhosis. Patients with hepatocellular carcinoma have increased release of cytokines, hormonal substances (GH, anabolic steroids) and altered tumor microenvironment (with hypercatabolic state, mutagenesis included by altered DNA, increased reactive oxygen species. Patients with HCC have underlying cirrhosis with hyperammonemia, decreased m-TOR activity which can contribute to sarcopenia. Non-tumor factors include poor nutrition and altered amino acid or lipid metabolism. HCC: Hepatocellular carcinoma; IL-1: Interleukin-1; IL-6: Interleukin-6; TNF-α: Tumor necrosis factor alfa; INF-γ: Interferon gamma; GH: Growth hormone; NF: Nuclear factor kappa B; STAT-3; Signal transducer and activator of transcription 3; ROS: Reactive oxygen species; NOS: Nitric oxide species; PGs: Prostaglandins; mTOR: Mechanistic target of rapamycin.

related to tumor progression by BCLC/TNM staging in HCC[33]. Follistatin is a glycoprotein and inhibitor of the TGF-β superfamily (such as myostatin, activin), and it can be related to tumor stage, size and can play an oncogenic role in hepatocarcinogenesis. These details provide important insights into potential agents such as myostatin inhibitors, mitochondrial protective agents, and antioxidants, which can be utilized for liver cirrhosis or HCC[55]. Such anti-sarcopenic treatments could be used to prolong or further reverse molecular, and metabolic changes noted in HCC patients.

CHANGES IN SARCOPENIA WITH HCC TREATMENTS

Sarcopenia in HCC patients undergoing various treatments (locoregional and systemic) has been shown to impact outcomes and survival. Multiple studies have reported outcomes among these patients. It has been showed that a baseline sarcopenia is associated with lack of response to HCC treatments, further decompensation episodes, and increased mortality[56]. In the following sections, we elaborate on studies evaluating the role of sarcopenia in HCC patients with various treatments such as loco-regional, surgery, transplant, and chemo/immunotherapy.

LOCO-REGIONAL THERAPY

Patients with HCC can be candidates for multiple loco-regional treatment (LRT) options such as radiotherapy, chemoembolization, radioembolization. Data on sarcopenic predicting response to LRT is sparse (Table 1)[14]. Iritani *et al*[15] reported 217 HCC patients on LRT and evaluated the role of sarcopenia. In this study, L3 skeletal muscle index (SMI) was used to define sarcopenia. Patients with low L3 SMI showed a significantly lower OS compared to those without sarcopenia (P = 0.004). Further, obese sarcopenic patients died earlier (P = 0.013)[15]. In 2015, Fujiwara *et al*[57] showed a higher risk of HCC recurrence in sarcopenic patients in 515 patients with BCLC stage 0/A who underwent percutaneous radiofrequency ablation (RFA). In 2017, a retrospective study of 182 patients with HCC undergoing percutaneous RFA therapy with curative intent was analyzed[58]. Patient with sarcopenia decreased pretreatment psoas muscle index (PMI) survival (overall cumulative survival) was

Table 1 Outcomes of hepatocellular carcinoma patients undergoing loco-regional therapy with sarcopenia

Ref.	Technique	*n*	Methods and outcomes
RFA			
Iritani *et al*[15] (2012-2014, Japan)	RFA	217	L3-SMI. B36.0 cm^2/m^2 for men and B29.0 cm^2/m^2 for women. Sarcopenia patients had lower OS than those without
Fujiwara *et al*[57] (2015, Japan)	RFA	515	L3-SMI used. B36.2 cm^2/m^2 for men and B29.6 cm^2/m^2 for women. Sarcopenia was associated with a higher risk of recurrence in very early/early-stage HCC who underwent treatment with RFA.
Yuri *et al*[58] (2017, Japan)	RFA	182	PMI used. 6.36 cm^2/m^2 for men and 3.92 cm^2/m^2 for women. Sarcopenia was associated with overall reduced HCC survival with no effect on recurrence.
TACE			
Dodson *et al*[38] (2013, United States)	TACE drug eluding TACE	216	TPA was used to assess sarcopenia. TPA of < 477 mm/m^2 for men and < 338 mm/m^2 for woman. Sarcopenia was independently associated with increased risk of death (lowest *vs* highest TPA quartile, HR = 1.84; $P = 0.04$)
Kobayashi *et al*[60] (2018, Japan)	TACE	102	L3-SMI used. 42 cm^2/m^2 for men and 38 cm^2/m^2 for women. Change in L3-SMI was an independent prognostic factor in patients with HCC treated with TACE.
Loosen *et al*[61] (2019, Germany)	TACE	56	Mean PMI was 11.81 mm/m^2. Low PMI (13.39 mm/m^2) had significantly lower median overall survival (491 d) compared to high PMI (1291 d)
Fujita *et al*[59] (2019, Japan)	TACE	179	PMI used. < 6.0 cm^2/m^2 for men and < 3.4 cm^2/m^2 for women. No difference was normal with low PMI and normal PMI for HCC outcomes. However, changes in PMI were significant after TACE with significant loss of liver function reserves post treatment.
TARE			
Faron *et al*[32] (2020, Europe)	TARE	58	MRI derived FFMA were used to predict sarcopenia. FFMA < 3582 mm^2 for men and < 2301 mm^2 for men. Low FFMA was associated with significantly reduced OS (197 *vs* 294, $P = 0.02$).

Studies depicting various loco-regional treatments utilized in hepatocellular carcinoma in relation to sarcopenia. RFA: Radiofrequency Ablation; TACE: Transarterial chemoembolization; TARE: Transarterial radiofrequency embolization; L3-SMI: Third lumbar vertebrae-skeletal muscle index; OS: Overall survival; HCC: Hepatocellular carcinoma; TPA: Total psoas area; FFMA: Fat-free muscle area; PMI: Psoas muscle index.

51.5% compared to 86.5% without sarcopenia ($P < 0.0001$. In addition to sarcopenia, total bilirubin ≥ 1.2 mg/dL, des-γ-carboxy prothrombin ≥ 34 mAU/mL ($P = 0.009$) were found to be adverse predictors of OS [58]. These findings were irrespective of CTP score or achievement of SVR in HCV-related HCC. Furthermore, above findings indicate the usefulness of sarcopenia to assess outcomes of HCC patients undergoing RFA.

Trans-arterial interventions for the HCC can be chemoembolization (TACE) or radioembolization (TARE) and are increasingly being utilized for large or multifocal disease with metastasis or macrovascular invasion[27]. The available data are conflicting about the role of sarcopenia as a predictor of survival in HCC who underwent TACE (Table 1). Fujita *et al*[59] and Kobayashi *et al*[60] showed no significant association between muscle volume at baseline and clinical outcomes. On the contrary, Loosen *et al*[61] and Dodson *et al*[38] showed that pre-interventional sarcopenia was associated with poor outcomes. Significant heterogeneity was noted in the methods to evaluate sarcopenia in these studies. The total psoas area (TPA), PMI, and L3-SMI were used to evaluate the presence of sarcopenia. If sarcopenia directed these effects (on the TACE efficacy) beyond the patients' general clinical condition or if this is mere an association, needs further evaluation in a prospective fashion. Data on the effects of sarcopenia on HCC patients with TARE is even more limited. Recently, Faron *et al*[32] reported 58 HCC patients using MRI-derived fat-free muscle area (FFMA) to predict sarcopenia. The FFMA < 3582 mm^2 for men and < 2301 mm^2 for women were used. In this study low FFMA was associated with significantly reduced OS (197 *vs* 294, $P = 0.02$)[32].

SURGICAL TREATMENTS

Liver resection

The role of sarcopenia in HCC patients undergoing liver resection is increasingly become topic of interest. Since, HCC patients often have poor nutritional status,

methods to reduce the catabolic state and improve protein synthesis, regeneration, Fan *et al*[62] investigated 124 patients to evaluate the role of nutrition in HCC resection. Nutrition therapy given prior to the liver resection with branched chain amino acids (BCAA), lipids, and dextrose have shown to decrease the worsening of liver function, sepsis-related complications, need for treatment for ascites, and overall decreased mortality. There was a reduction in the overall post-operative morbidity in the nutrition group compared to the control group (34% *vs* 55%; relative risk, 0.66; 95%CI: 0.45-0.96)[62]. In 2013, Harimoto and colleagues[63] studied 186 HCC patients with sarcopenia using L3-SMI (< 43.75 for men, < 41.10 for women), and a significant correlation was noted between sarcopenia and liver dysfunction (indicated by low albumin levels and indocyanine green retention). In patients with and without sarcopenia, the 5-year OS rate was 71% and 83·7%, and the 5-year recurrence-free survival rate was 13% and 33·2%, respectively[63]. Additionally, studies evaluated the relationship between total functional liver volume (TFLV) and sarcopenia (L3-SMI) and found that median TFLV was significantly lower in the sarcopenic group than the normal group (1296 mL *vs* 1840 mL; $P < 0.05$)[64].

Sarcopenic obesity characterized by increased fat volume compared to skeletal muscle mass. As obesity and loss of muscle share common pathophysiological mechanisms, combined insult could display a poor outcome. Studies evaluated the effect of sarcopenic obesity in HCC and found that patients with sarcopenic obesity had worse median survival (84.7 mo *vs* 39.1 mo, $P = 0.002$) and worse median recurrence-free survival (21.4 mo *vs* 8.4 mo, $P = 0.003$)[21]. Additionally, it was identified as an independent risk factor for death and HCC recurrence[21]. Effect of sarcopenia on immediate and short-term clinical outcomes after hepatic resection was examined by Otsuji *et al*[65] Sarcopenic patients had a higher postoperative length of stay, higher rates of liver failure, major complications, and intra-abdominal abscess formation (Table 2). Multiple other studies have provided similar results with different modalities to evaluate sarcopenia, such as L3-SMI, TPA, and visceral-to-subcutaneous adipose tissue ratio (Table 2). Furthermore, these studies used differing SMI cut-off points to define sarcopenia. The majority of the studies point to poor outcomes in patients with sarcopenia, which might be due to the underlying liver dysfunction and HCC severity. Nevertheless, prospective data with uniform cut-off points to assess SMI to define sarcopenia in these studies to provide concrete evidence of the relationship between sarcopenia and liver resection.

Liver transplantation

Sarcopenia in patients awaiting liver transplantation (LT), perioperative and postoperative outcomes have been studied recently[66-71]. Multiple methods to assess sarcopenia were used (Table 3). For example, L3-SMI, psoas muscle thickness, MELD-sarcopenia score, skeletal muscle mass-to-visceral fat area ratio (SVR), TPA, PMA, and height normalized psoas muscle thickness were used. Among these, L3-SMI is the most commonly used objective way of assessing sarcopenia. Further, studies evaluated the wait times and survival related to sarcopenia (Table 3).

Studies performed on outcomes in LT patients evaluated the preoperative status of the patients (listed and waiting for the transplant), procedural outcomes and post-procedure long-term survival. Carey *et al*[68] in 2016 used L3-SMI with 50 cm^2/m^2 for men and 39 cm^2/m^2 for women and noted that individuals who died had lower SMI compared to those who survived (45.6 cm^2/m^2 *vs* 48.5 cm^2/m^2; $P < 0.001$), and SMI was associated with wait-list mortality (HR, 0.95; $P < 0.001$). Wada *et al*[67] in 2017 considered sarcopenia for TPA of 791.6 mm^2/m^2 for men and 488.8 mm^2/m^2 for women. The authors compared TPV to TPA. The preoperative total psoas volume (TPV) was found to be a better predictor than TPA in assessing post-operative risks in living-donor LT recipients[67]. Multiple studies evaluated the LT outcomes and complications such as infections, length of stay, failure to rescue, and surgery-related events[72,73]. The rate of infections was assessed and compared to individuals with sarcopenia. Patients with sarcopenia had a higher prevalence of sepsis, bacterial pneumonia, longer ICU stays, and mortality[2,69]. Postoperative survival was studied by Van Vugt *et al*[69] and Kaido *et al*[72] who noted that sarcopenia was inversely associated with clinical outcomes after LT. Few studies noted sarcopenia developing after the LT, which is probably due to underestimation of muscle mass/strength estimation before LT. In addition to underlying cirrhosis, increased catabolism, tumor-related morbidity noted in these patients, the role of immunosuppressant use cannot be underestimated. The use of mTOR and calcineurin inhibitors can potentially lead to sarcopenia[74]. Further, renal dysfunction caused by calcineurin inhibitors can compound these effects. The results of these studies provide an opportunity for improving the nutritional status in sarcopenia LT patients with dietary and exercise

Table 2 Outcomes of hepatocellular carcinoma patients undergoing liver resection (hepatectomy) with sarcopenia over last 5 years

Ref.	Technique	*n*	Methods and outcomes
Otsuji *et al*[65] (2015, Japan)	Major hepatectomy and extrahepatic bile resection	256	Total psoas area (TPA) was used to assess sarcopenia. TPA of < 567 mm/m^2 for men and < 395 mm/m^2 for woman. Length of postoperative hospital stay were longer (39 d *vs* 30 d, P < 0.001, high rate of liver failure (33% *vs* 16%), major complications (54% *vs* 37%), intra-abdominal abscess (29% *vs* 18% compared to those without sarcopenia (P < 0.05)[69].
Voron *et al*[110] (2015, Japan)	Hepatectomy	198	L3-SMI used 52.4 cm^2/m^2 for men and 38.9 cm^2/m^2 for women. Sarcopenia was associated with shorter median OS (52.3 mo *vs* 70.3 mo; P = 0.01 and it was an independent predictor of OS and DFS.
Yabusaki *et al* [111] (2016, Japan)	Primary hepatectomy	195	SMI used 43.75 cm^2/m^2 for men and 41.10 cm^2/m^2 for women. Sarcopenia was associated with poor cumulative recurrence rate (P = 0.13).
Takagi *et al*[113] (2016, Japan)	Curative hepatectomy	254	L3-SMI used 46.4 cm^2/m^2 for men and 37.6 cm^2/m^2 for women. The sarcopenic group had a significantly lower 5-yr OS rate than the non-sarcopenic group (58.2% *vs* 82.4%, P = 0.0002). Further it was an independent predictor of poor survival (HR =2.28, P = 0.002) and poor ASA status (HR = 3.17, P = 0.001).
Kobayashi *et al* [21] (2019, Japan)	Hepatectomy	465	L3-SMI used. 40.31 cm^2/m^2 for men and 30.88 cm^2/m^2 for women. Sarcopenic obesity as a significant risk factor for mortality (HR = 2.504, P = 0.005) and recurrence of HCC (HR = 2.031, P = 0.006) after hepatectomy for HCC.
Hamaguchi *et al* [112] (2019, Japan)	Hepatectomy	606	L3-SMI was used to assess the sarcopenia. SMI of < 40.31 for men and 30.88 for women were used. A high visceral-to-subcutaneous adipose tissue ratio, low SMI, and high IMAC contributed to an increased risk of death (P < 0.001) and HCC recurrence (P < 0.001) in an additive manner.
Xu *et al*[22] (2020, China)	Hepatectomy	1420	Authors performed a meta-analysis of six studies and preoperative sarcopenia was significantly associated with poor OS (HR =1.58, 95%CI: 1.34-1.84, P = 0) and shorter DFS (HR =1.54, 95%CI: 1.17-2.02, P = 0.002) in patients with HCC undergoing hepatectomy[24].

Studies, techniques and outcomes to evaluate the success of liver resection in patients with sarcopenia and hepatocellular carcinoma. L3-SMI: Third lumbar vertebrae- skeletal muscle Index; OS: Overall survival; SMI: Skeletal muscle index; HR: Hazards ratio; DFS: Disease free survival. HCC: Hepatocellular carcinoma; TPA: Total psoas area; IMAC: Intramuscular adipose tissue content; PMI: Psoas muscle index.

measures during pre, peri and post-operative period.

Systemic therapies

The use of chemotherapy and immunotherapy has become the mainstay of treatment for HCC lesions that are not amenable to LRT or LT. Sorafenib is the most studied and prescribed chemotherapeutic agent in HCC[75]. Although it can prolong survival, its use is limited by its adverse effects such as nausea, excessive fatigue, and diarrhea noted in most patients. These studies evaluated multiple outcomes such as OS, progression-free survival, mortality were evaluated in different studies in HCC patients receiving Sorafenib therapy[76-82]. While the ways to assess the sarcopenia differed in these studies, most commonly used method is L3-SMI. Further various cut-off values were utilized in these studies.

Nishikawa *et al*[78] studied 232 patients to evaluate for OS using L3-SMI. The authors noted that the patients with sarcopenia had significantly low median OS of 174 d compared to 454 d in the non-sarcopenic group (P < 0.0001). Multivariate analysis showed that sarcopenia was an independent predictor of OS. Similarly, Takada *et al*[81] studied 214 patients in which OS in pre-sarcopenia patients were worse than without pre-sarcopenia (median 252 d *vs* 284 d, respectively; P = 0.16). Saeki *et al*[82] reported 100 advanced HCC patients using use of L3-SMI showing individuals without muscle depletion had longer survival was noted (HR = 0.50, P = 0.006). This combined with low tumor number (< 7) and lack of extrahepatic spread offered better survival in these patients[82]. Dynamic assessment of sarcopenia has assisted to compare outcomes before and after starting sorafenib. Few studies noted that sarcopenia worsened after the initiation of sorafenib. If this is due to the progression of HCC or angiogenic (or Carnitine inhibitory) properties of sorafenib needs further evaluation[83]. Further, Cheng *et al*[34] reported that pre-sarcopenia could independently predict the outcomes in sorafenib-failed HCC.

Use of other modalities such as fat mass indices (visceral, subcutaneous) in combination with L3-SMI and their relative changes (over a period of time) can assist in assessing sarcopenia and can predict outcomes in HCC patients receiving sorafenib [82]. However, more studies are needed to confirm these findings. Recently newer agents for HCC are increasingly utilized such as Regorafenib, Lenvatinib, Nivolumab, the combination of gemcitabine and oxaliplatin (GEMOX regimen)[30,84-86]. Studies showing the effect of sarcopenia on HCC patients' survival using these agent are

Table 3 Outcomes of hepatocellular carcinoma patients undergoing liver transplant with sarcopenia over last 5 years

Ref.	Technique	*n*	Methods and outcomes
Itoh *et al*[114] (2016, Japan)	Living-donor LT	153	Based on SVR, patients with low SVR were had poor prognosis than without low SVR for OS (P = 0.03) and recurrence-free survival (P = 0.01).
Carey *et al*[68] (2016, United States)	Awaiting LT	396	L3-SMI used. 50 cm^2/m^2 for men and 39 cm^2/m^2 for women. Patients who died had lower SMI compared to those who survived (45.6 cm^2/m^2 *vs* 48.5 cm^2/m^2; P < 0.001), and SMI was associated with wait-list mortality (HR, 0.95; P < 0.001)[72].
Wada *et al*[67] (2017, Japan)	LDLT	32	TPA was used. TPA of 791.6 mm^2/m^2 for men and 488.8 mm^2/m^2 for women. TPV was used to compare to TPA. Preoperative TPV is a better predictor compared to TPA in assessing post-operative risks in LDLT recipients[71].
Golse *et al*[70] (2017, Europe)	LT	256	PMA, L3-SMI was used. 1561 mm^2 for men and 1464 mm^2 for women. One and 5-yr OS rates were significantly poorer in the sarcopenic group than in the nonsarcopenic group at 59% *vs* 94% and 54% *vs* 80%, respectively (P < 0.001). Authors concluded that pre-LT PMA might be predict 1-yr survival post-LT[74].
Van Vugt *et al*[69] (2017, Europe)	Listed for LT	585	L3-SMI used. 43 to 53 cm^2/m^2 for men based on the BMI and 41 cm^2/m^2 for women. Sarcopenia was associated with waiting list mortality in liver transplant candidates with cirrhosis, particularly in patients with lower MELD scores (P < 0.001) [73].
Kim *et al*[71] (2018, Japan)	LDLT	92	Height normalized psoas muscle thickness (< 15.5 mm/m) at L3. HCC recurrence risk was greater in sarcopenic patients in univariable analysis [HR = 8.06 (1.06–16.70), P = 0.044) and in multivariable analysis [HR = 9.49 (1.18–76.32), P = 0.034][75].
Chae *et al*[66] (2018, South Korea)	LDLT	408	This study investigated the association between a perioperative decrease in the PMI and patient mortality after LT. A PMI decrease ≤-11.7% between the day before surgery and POD-7 was an independent predictor of patient mortality after LT[70].

Techniques, methods and outcomes to evaluate the success of liver transplantation in patients with sarcopenia and hepatocellular carcinoma. LT: Liver transplant; LDLT: Living-donor LT; SVR: Skeletal muscle mass-to-Visceral fat area ratio; TPV: Total psoas volume; PMA: Psoas muscle area; BMI: Body mass index; L3-SMI: Third lumbar vertebrae- skeletal muscle index; OS: Overall survival; SMI: Skeletal muscle index; HR: Hazards ratio; DFS: Disease free survival; HCC: Hepatocellular carcinoma; TPA: Total psoas area; PMI: Psoas muscle index.

sparse. Lenvatinib induces minimal muscle loss after 2 years of treatment correlates with its low toxicity[23,30,87]. Combined effects of sarcopenia and inflammation (by high neutrophil-to-lymphocyte ratio and absolute lymphocyte count) have been studied in patients receiving nivolumab in HCC patients[28]. If inflammatory markers are more important than sarcopenia in patients received immunotherapy needs further validation[25,28]. Overall, sarcopenia can predict survival in advanced HCC patients receiving chemotherapeutics such as sorafenib before initiation of the treatment and during and after the treatment. Strategies to improve the muscle mass, nutrition can add to the survival in these patients. Further studies are needed to evaluate the role of sarcopenia for new chemotherapy and for immunotherapy.

METHODS TO IMPROVE SARCOPENIA

As sarcopenia can adversely affect the outcomes of HCC patients undergoing treatments, methods to improve could impact the survival of these patients. As HCC happens with a background of cirrhosis in up to 80%-90% of patients, improving sarcopenia in cirrhotics could assist in improving survival. Reversing pathophysiology by improving myofibres size, number, reversing myosteatosis, inhibiting mitochondrial integrity loss, mTOR signaling, and decreasing ROS accumulation can improve sarcopenia in both HCC and cirrhotics. Two major strategies exist to improve sarcopenia in these patients- nutritional support and physical exercise. Use of L-carnitine, BCAA, leucine have been used in the studies to increase the nutritional component[88,89]. Improvement of skeletal mass (PMI) was noted after the supplementation of these agents in these studies. Physical exercise can recruit more myofibres and at least inhibit sarcopenia. It is unclear if it can reverse the sarcopenia completely. Both isometric (lifting hand weights 2-3 times per week) and isotonic (30-40 min walking 3-4 times per week) have been used to improve muscle strength in these patients[90-94]. Studies have shown an increased muscle cross-sectional area (quadriceps) with exercise in cirrhotics of at least 10%[95]. Although, testosterone supplementation have been reported to improve the sarcopenia, few reports of alpha-alkylated formulation could theoretically increase the risk of HCC formation[96].

The role of non-steroidal Selective Androgen Receptor Modulators (SARMs) is increasingly being recognized in the treatment of sarcopenia[97-99]. SARMs inhibit protein degradation and thereby could decrease the rate of sarcopenia. Multiple animal models have been used to evaluate mechanisms of SARMs to reverse muscle atrophy in degonadized mice. For instance, SARM treatment in ovariectomized rat model can increase muscle mass by enhanced mitochondrial biogenesis, actin and myosin[98]. SARMs can target androgen receptors and decrease sarcopenia *via* paracrine growth factor signaling on vimentin positive muscle fibroblasts[97]. Further, upregulation of mTOR, glycogen synthase kinase[99]. SARMs also exhibit anabolic effects, increasing the bone and muscle mass which are affected in patients with HCC. A combination approach of nutritional supplementation with physical exercise with a multidisciplinary approach has been tried in cirrhotics and HCC patients[31]. Significant changes in muscle volume was noted after the intervention[95]. Similarly, a combined approach has been tried in a few studies in HCC patients undergoing TACE [100,101]. This approach has been studied in patients waiting or LT, with good response[102,103]. In conclusion, a combined multidisciplinary approach is useful and logical to improve the sarcopenia in cirrhotics and HCC which might eventually improve outcomes of these patients undergoing local, surgical and systemic therapies.

FUTURE DIRECTIONS

Although sarcopenia can offer significant details about the functional status, it can be further enhanced by the use of frailty (using clinical frailty scale, liver frailty index, Karnofsky performance status) and amount of malnutrition (by assessment of BMI, nutritional intake). These can be incorporated into composite scoring to better evaluate the functional status of HCC patients. Recently use of changes in bone resorption *via* upregulation of inflammatory cytokines opened the concept of sarcopenic osteoporosis [104]. A crosstalk between skeletal muscle, bone homeostatic changes with underlying cirrhosis and HCC can provide pathways for treatments in the future. Myostatin, irisin, osteocalcin, activation of Wnt/β-catenin pathways have been implicated in sarcopenic osteoporosis. Furthermore, biomarkers such as imbalance of plasma free amino acids (BCAA) have been implicated in progression of HCC[105]. If this could be a reliable way to improve the sarcopenia in HCC patients remains to be studied.

Precision medicine tools such as use of radiomics and radiogenomics are emerging for assessing host and tumor-related risk factors in HCC[106,107]. Radiomics uses medical imaging data to develop reproducible quantitative data from qualitative images. This has been utilized for lung cancer assessment of tumor and non-tumor tissue[108]. Development of methods to quantify the amount of normal non-tumor liver tissue in HCC patients is essential for surgeons to evaluate resection strategies. Seror *et al*[109] noted that use of non-invasive cross-sectional imaging to assess the liver surface nodularity and lean body mass can act as surrogate markers for liver cirrhosis and sarcopenia. Patients with higher liver surface nodularity (OR 7.05, 95%CI: 2.13-23.25) and sarcopenia (OR 6.51, 95%CI: 2.08-20.39) were associated with high risk of complications[109]. A step further in this direction, use of genomics (cellular and molecular changes) to existing radiomics can provide radiogenomic information which can be used to develop molecular signatures for development for actionable clinical targets[107]. Finally use of artificial intelligence and deep learning can lead to next generation biostatistical and informatic data to develop algorithms and pathways to identify optimal clinical patterns[106].

CONCLUSION

Sarcopenia is increasingly recognized as a predictive marker for assessing outcomes in HCC patients. There is increasing evidence to evaluate its role in loco-regional, surgical, transplant, and systemic treatment options in HCC patients. Early recognition to identify sarcopenia, methods to improve the muscle volume, strength, and mass could impact the patient outcome and OS. The use of appropriate nutritional support, physical activity or both could potentially improve muscle volume in these patients. However, it is unclear about the degree of improvement of the sarcopenia with all of these measurement combined. Further, prospective studies aimed at interventions that could potentially reverse sarcopenia to improve HCC patients' outcomes are needed in the future.

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MINIREVIEWS

Celiac disease: From genetics to epigenetics

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Abstract

Celiac disease (CeD) is a multifactorial autoimmune disorder spread worldwide. The exposure to gluten, a protein found in cereals like wheat, barley and rye, is the main environmental factor involved in its pathogenesis. Even if the genetic predisposition represented by HLA-DQ2 or HLA-DQ8 haplotypes is widely recognised as mandatory for CeD development, it is not enough to explain the total predisposition for the disease. Furthermore, the onset of CeD comprehend a wide spectrum of symptoms, that often leads to a delay in CeD diagnosis. To overcome this deficiency and help detecting people with increased risk for CeD, also clarifying CeD traits linked to disease familiarity, different studies have tried to make light on other predisposing elements. These were in many cases genetic variants shared with other autoimmune diseases. Since inherited traits can be regulated by epigenetic modifications, also induced by environmental factors, the most recent studies focused on the potential involvement of epigenetics in CeD. Epigenetic factors can in fact modulate gene expression with many mechanisms, generating more or less stable changes in gene expression without affecting the DNA sequence. Here we analyze the different epigenetic modifications in CeD, in particular DNA methylation, histone modifications, non-coding RNAs and RNA methylation. Special attention is dedicated to the additional predispositions to CeD, the involvement of epigenetics in developing CeD complications, the pathogenic pathways modulated by epigenetic factors such as microRNAs and the potential use of epigenetic profiling as biomarker to discriminate different classes of patients.

Key Words: Celiac disease; Epigenetics; DNA methylation; Histone modifications; Long non-coding RNAs; MicroRNAs

Core Tip: Currently identified genes account only for half of celiac disease (CeD)

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predisposition. An important role could be played by epigenetics, inheritable traits without DNA sequence alterations, which could be influenced by gluten exposure. DNA methylation, histone modifications and non-coding RNAs act on different gene expression steps, from gene transcription to post-translational ones. Epigenetic changes can be additional predisposition factors or specific of CeD stages (active disease, gluten-free diet) as recently reported. Analysis of epigenetic data and their integration with transcriptome (by machine learning) can help to stratify patients, or discover new players in CeD pathogenesis, possible focus of novel therapeutic approaches.

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INTRODUCTION

Celiac disease (CeD) is a multifactorial autoimmune enteropathy that develops in genetically predisposed subjects carrying the HLA-DQ2 or HLA-DQ8 haplotype. The prevalence of these HLA haplotypes in the general population is around 30%-40%, suggesting that they are necessary, but not sufficient, to induce CeD[1]. Regional variancies have been observed in the frequency of these haplotypes in CeD, with the HLA-DQ8 that ranges from about 2%-4% in western countries to 25%-30% in the Iranian population and in the middle East[2]. Indeed, CeD prevalence worldwide is about 1%-2%, with environmental factors that contribute to the regional differences, like the exposure to gluten, the known CeD exogenous antigen[1]. The clinical presentation of CeD is quite heterogeneous, ranging from classical intestinal-related symptoms (diarrhoea, failure to thrive) to non-intestinal manifestations (anaemia, dermatitis, osteoporosis), and such diverse clinical picture may delay, in some cases, the prompt diagnosis[3]. Even if the main mechanisms by which gluten peptides cause CeD intestinal lesion are now quite established, the reason why only a few of the genetically predisposed subjects develop the disease still needs to be clarified. In fact, geneticists calculated that the presence of a specific HLA accounted only for about 40% of the genetic predisposition, leaving most of the genes involved in the development of the disorder still unknown[4]. Thus the initial focus of researchers was to identify further genes that could constitute a "genetic background" predisposing to the disease. Few studies used the classical linkage analysis approach, but the obtained results were specific of a single population and could not be replicated[5], or the identified genomic region did not harbour genes that appeared to be involved in CeD pathogenesis[6]. The identification of additional predisposing genes was obtained using a different approach, *i.e.*, Genome Wide Association Studies (GWAS) performed on populations of different geographical origin. These studies identified 39 Loci associated with CeD development, but also confirmed the role of the Human Leukocyte Antigen (HLA) region. Additional analyses revealed that some loci included more than one gene associated with CeD, thus raising the number of the involved polymorphisms (single nucleotide polymorphisms, SNPs) up to 57. Although these studies were quite extensive, the identified loci were not able to completely explain CeD genetic predisposition, since HLA + all these additional genes accounted for about 54% of the hereditability of CeD[7]. Interestingly, a great number of these SNPs involved genes with an immune function either in the intestine or in the thymus, further supporting the idea that an alteration of the immune response represents an essential step in CeD predisposition and pathogenesis. Moreover, a more recent paper was able, integrating genomic and transcriptomic data, to prioritize genes involved in CeD, and to identify TRAF-type zinc finger domain containing 1 (TRAFD1) as a master regulator of genes involved in interferon (IFN)γ signaling and MHC I antigen processing/presentation[8]. The different combination of these SNPs could also be associated with a different phenotype, as recently reported by Cerquieira *et al*[9], who identified the TLR7/ TLR8 Locus associated with disease onset before 7 years of age, whereas SH2B3/ATXN2, ITGA4/UBE2E3 and IL2/IL21 Loci were associated with later development of CeD and a more severe small bowel mucosal damage. In addition, SH2B3/ATXN2 was associated with type 1 diabetes; in fact some of the

identified SNPs are described as predisposing factors also for other autoimmune disorders like type 1 diabetes and Crohn's disease[7,10], almost suggesting the presence of a common genetic background predisposing to autoimmunity. It must also be noted that, among the identified SNPs, only 5% were present in coding regions, and about 5% and 9% in 5' and 3' untranslated regions, respectively[7]. This means that 81% of the identified SNPs were either in intergenic or intronic regions, suggesting that their role could be to regulate gene expression, possibly through the interaction with transcription factors or proteins able to regulate chromatin status, *i.e.*, epigenetic modifications. Epigenetics has gained much attention in recent years, but its involvement in CeD still needs to be well characterized. First of all, this term has been used to define a wide range of concepts, and the 2008 Cold Spring Harbor meeting defined epigenetics as *"the study of a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence"*[11]. DNA methylations, histone modifications resulting in chromatin remodelling and non-coding RNAs are the three main categories that fall into this statement. The latter one also includes microRNAs, that have been found deregulated in CeD patients, suggesting that the regulation of the expression of target mRNAs by post-transcriptional modifications is relevant in CeD pathogenesis[12-14]. In this direction, RNA sequencing studies are currently trying to identify different gene expression signatures that could help stratify patients based on CeD stage/presentation, or highlight new pathways implicated in CeD development[15-17].

In this review we will provide an update on the current knowledge about epigenetics in CeD and investigate the possible role of epigenetic profiling in patient classification and/or in determining the risk in subjects with familiarity to CeD.

LITERATURE SEARCH

The literature search was carried out on PubMed, searching the key words "epigenetics", "histones -acetylation, -methylation, -phosphorylation, -ubiquitination", "chromatine", "non-coding RNA", "lncRNA", "miRNA", "microRNA", "RNA methylation" AND "celiac disease". The reviewed papers were original research articles published from 2010 to October 2021. All articles that were considered relevant to the purpose of the present review were included, whereas the ones that did not add novelty or did not give clear results were excluded during the critical revision of the literature.

DNA METHYLATION

DNA methylation is the most known epigenetic modification, that sees the family of DNA methyltransferase (DNMTs) enzymes transfer methyl-groups to the C-5 residue of cytosine pyrimidine ring. This phenomenon occurs in particular in CpG-rich regions (CpG islands), mainly localized in promoters and regulatory regions. Being the methylated DNA physically less accessible for transcription, this causes the inactivation of the nearby genes[18]. The majority of gene promoters are normally methylated to guarantee a fine regulation of transcription in different tissues or cell types. Like most of the epigenetic modifications, it does not directly alter the DNA sequence, but a typical methylation profile can usually be inherited. Hypo-methylation or hyper-methylation of specific genomic loci has been reported to predispose to disease and cancer[19]; for this reason, the first studies on DNA methylation in CeD analyzed the predisposition to develop small bowel adenocarcinomas. In particular, Bergmann *et al*[20] found a high level of CpGs methylation and microsatellite instability correlated to the loss of MLH1 expression in three different small bowel carcinomas in CeD patients, whereas this feature was not present in non-CeD patients. A similar finding was observed by Diosdado *et al*[21], who also detected an hyper-methylation of the *APC* gene promoter that caused defects in the mismatch repair mechanisms in these patients. Further studies were conducted by Rizzo *et al*[22], who were able to identify four different CpG Island Methylator Phenotypes, with two of them being specific for CeD patients.

Although these data highlight a typical methylation pattern in CeD complications, the identification of a methylation profile associated with the predisposition for developing CeD is essential. Since GWAS pointed out the presence of many risk variants common to other autoimmune diseases, it has been hypothesised that they could also share common methylation patterns. Hammaker *et al*[23] focused on the

specific risk variant rs906868, shared between CeD and rheumatoid arthritis and mapping in the promoter of LBH, gene with a regulatory function on the Wnt pathway, essential for differentiation and development. Interestingly, the authors detected a differential methylation pattern in the two diseases, thus suggesting the possibility of a disease-specific profile[23]. Methylation can also occur only on one allele, thus not causing a complete silencing of the gene but its modulation. Hutchinson *et al*[24] investigated allele-specific methylation (ASM) on CpG islands localized throughout the genome, detecting four of them as implicated in complex diseases. In particular, rs2762051, a C/T risk variant that maps in the non-coding RNA DLEU1, undergoes ASM in CeD[24]. It is interesting to note that this variant belongs to the group of SNPs previously identified as significantly associated to CeD predisposition[7], thus further supporting the need for an integration between the genetic and epigenetic profile. A specific gene methylation could also be associated to a different phenotype, in particular if the methylation site is able to influence the expression of disease-related proteins. In fact, a lower level of methylation was detected in different IFNγ CpGs in patients with ulcerative colitis (UC) and a more severe phenotype[25], suggesting that methylation can regulate this UC pivotal pathogenetic pathway. Fernandez-Jimenez *et al*[26] demonstrated that this can also happen in CeD, since they observed not only a different methylation profile in the genes of the core NFkB pathway in active CeD subjects as compared to controls, but also an inter-mediate pattern in CeD patients on gluten-free diet (GFD). Although it was not clear if gliadin could directly affect the methylation of these specific genes, long exposure to gliadin could be able to affect the methylation pattern, since proline-rich peptides seem to act as opioid-like molecules causing a modulation in glutathione activity and DNA methylation[27].

It is also known that methylation profiles can be cell type-specific, thus a single cell methylation analysis could provide essential information. However, the methods employed to obtain single cell preparations can sometimes alter the DNA methylation patterns as described by Jenke *et al*[28], thus pointing out the importance of considering this aspect in designing this kind of studies. Another potential bias of methylation studies regards the preferential amplification of an allele or a strand, based on its methylation degree. For this reason Ochoa *et al*[29] realized a new Bayesan calibration method and validated it also on CeD patients samples, and the most recent studies on methylation in CeD considered these techniques. Fernandez-Jimenez *et al*[30] compared the whole methylome of the epithelium and immune cells from CeD and controls biopsies, finding a cell-specific methylation pattern, with 43 (11 hypo-, 32 hyper-methylated) and 310 (40 hypo-, 270 hyper-methylated) differentially methylated positions (DMP) in the epithelium and in immune cells respectively. It is important to note that only a portion of the DMPs was shared between the two cellular components, highlighting a cell-specific, disease-driven modification. Cielo *et al*[31] used a different approach, separating the epithelium from the lamina propria and analysing the expression and methylation of genes known to be altered in CeD. Among the candidate genes, they found a decreased methylation in SB2H3 and IL21, that coupled with their increased expression in the epithelium and lamina propria respectively, but only in active CeD compared to controls. Since these genes take part in the pathways mainly involved in inflammation and barrier integrity in CeD, identifying a different signature in patients and controls may partially explain the abnormal response to food antigens and help patient stratification[31]. Finally, the most recent study in this field by Hearn *et al*[32] compared methylation profiles in saliva samples obtained from CeD patients on a GFD and controls with the use of a beadchip array, finding a different methylation pattern in HLA-DQB1 and in specific loci near *SLC17A3* gene in a pilot cohort, data not confirmed in a larger study group. Being this collection method non-invasive, it could be a great tool for patient discrimination, especially in the screening of predisposed subjects, once a different and discriminatory methylation profile has been clearly established. Moreover, the patient histological classification needs to be taken into account, even if a partial concordance in methylation patterns in saliva and intestine has been observed in a small group of patients[33]. In this direction, encouraging results come from studies on inflammatory bowel diseases (IBDs) from Howell *et al*[34], that were able to statistically discriminate different classes of patients by analysing the transcriptome and the methylation status in the intestinal epithelium, correlating them with the disease outcome. Indeed, methylation profiling can be an asset to study different aspects of CeD, both to understand its pathogenesis and as a biomarker. A graphical representation of DNA methylation is present in Figure 1, whereas its highlights in CeD are reported in Table 1.

Table 1 DNA methylation features in celiac disease

Predisposition to CeD	What	Result	Highlight in CeD
Allele-specific methylation (ASM) [24]		ASM in rs2762051 in *DLEU1* gene	Linked to CeD phenotype
Rs906868 in *LBH* gene promoter [23]	Risk variant shared with RA	Disease-specific methylation	Different methylation in rs906868 can predispose to CeD or RA → influence on Wnt signalling
Methylation in HLA region in CeD[30]	Specific patterns in epithelial and immune cells	Genotype-independent methylation (except for HLA-DPB2)	Methylation patterns in HLA region → CeD predisposition
Methylation profiling in HLA-DQB1 and SLC17A3[32]	Bead-chip on saliva samples	Different methylation profiles, not confirmed in bigger cohort	Potential methylation-based screening Major validation needed
Opioid like-effect of gliadin[27]		Modulation of glutathione and DNA methylation	Predisposition to inflammation and oxidation
CeD pathogenesis			
Methylation in NFkB-related genes[26]	NFkB pathway↑↑	Disruption of regulatory equilibrium	Co-methylation patterns typical of active CeD in NFkB pathway genes
Cell-specific methylation[30]		Epithelium → 43 DMP Immune cells → 310 DMP	Cell-specific methylation signature & gene expression in CeD *vs* controls
Different methylation of SB2H3, IL-21, cREL and TNFAIP3[31]	Epithelium and lamina propria - specificity	Correlation with pro-inflammatory(↑) and cell adhesion(↓) pathways	Methylation of SB2H3 → epithelium Methylation of IL21 → lamina propria Typical of CeD samples.
Tumor development			
↑CpGs methylation[20]	Microsatellite instability	↓MLH1 expression	Typical in CeD-related small bowel adenocarcinoma
MLH1 deregulation[21]	*APC* gene hyper-methylation	Chromosomal aberrations/microsatellite instability	Defects in mismatch repair Typical in CeD-related small bowel adenocarcinomas
Microsatellite instability[22]	Typical CpGs methylator phenotype	Methylation profiling → phenotypical classification	Mesenchymal and immune phenotypes are common in CeD-related small bowel carcinoma

CeD: Celiac disease; ASM: Allele specific methylation; RA: Rheumatoid arthritis; DMP: Differentially methylated positions; CpGs: CpG islands.

HISTONE MODIFICATIONS

A further step in gene expression regulation in the nucleus is represented by histone modifications. Histones are key proteins of chromatin, able to modify its structure making the DNA more or less accessible to transcription. The most common histone modifications are acetylation, phosphorylation, ubiquitination and methylation. Gene expression analysis studies performed on CeD biopsies compared to controls, pointed out a differential regulation of histone-modifying enzymes, thus suggesting the presence of a disease-related epigenetic signature involving histone modifications[16, 17].

Histone acetylation, governed by acetyltransferase (HATs) and deacetylase (HDACs) enzymes, usually result in active gene transcription due to chromatin relaxation[35]. Zorro *et al*[36] analyzed the transcriptomic and epigenetic responses to IL-15, IFNβ and IL-21 stimulation in primary cytotoxic T lymphocytes derived from CeD biopsies. Specific transcriptomic patterns were identified for the different cytokine stimulation, but also different levels of histone acetylation (H3K27ac) were detected. Interestingly, the increase in transcription after IFNβ stimulation was associated, in about 60% of the cases, with an increase in H3K27ac in promoter and enhancer regions, whereas this was not the case after IL-15 stimulation, pointing towards the presence of a different regulatory mechanism. In fact, upon IL-15 stimulation a relevant number of differentially expressed genes were ncRNAs, suggesting their potential regulatory role independently from H3K27ac modifications

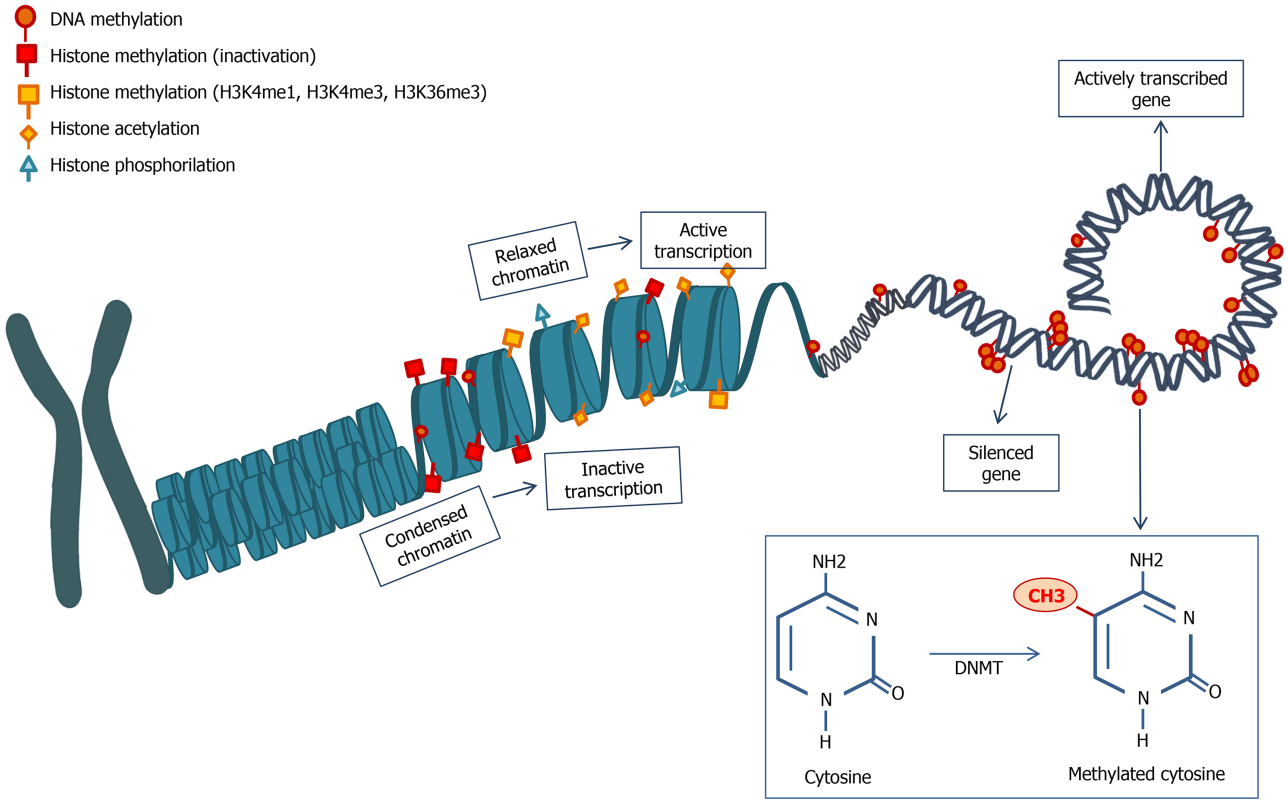

Figure 1 Schematic representation of DNA methylation and histone modifications. Histone modifications are many and determine a different chromatin status, modifying DNA accessibility and interfering with gene transcription. Methylation can also happen directly on the DNA sequence, mainly resulting in gene silencing. The image is original and was created with the use of Servier Medical Art modified templates, licensed under a Creative Common Attribution 3.0 Unported License (https://smart.servier.com). DNMT: DNA methyltransferase.

[36].

Histone methylation is more complex and mainly linked to repression, but the specific methylations H3K4me1, H3K4me3 and H3K36me3 are commonly found in actively transcribed regions[35]. In fact, H3K4me3 was used as a marker of active transcription by Gutierrez-Achury *et al*[37]; the authors firstly identified SNPs associated with both CeD and rheumatoid arthritis, but also SNPs specific for each disease. The combination of these data with H3K4me3 profiles available in public databases allowed the authors to detect similar histone enrichment corresponding to the common SNPs in less specialized immune cell types, whereas disease-specific SNPs overlapped with H3K4me3 profiles in more specialized cells[37]. H3K36me3 signature was investigated by Moffitt *et al*[38] in the enteropathy associated T cell lymphoma (EATL), the most common neoplastic consequence of CeD. The histone-lysine N-methyltransferase SETD2 is the responsible for this kind of methylation and it was silenced in 32% of EATL in CeD subjects. In fact, *in vivo* studies with a knock out model for SETD2 in T cells carried a decreased H3K36me3 pattern along with γδ-T cells expansion[38]. H3K27me3 was instead used as gene silencing signature by Oittinen *et al*[39] in determining the involvement of the polycomb repressive complex 2 (PRC2) in controlling Wnt signalling in the intestine. They found a H3K27me3 pattern which differed according to the cell position/differentiation along the crypt/villus axis, and involved also genes related to proliferation and differentiation in the epithelium. Thus PRC2-driven tri-methylation is important to maintain the homeostasis driven by Wnt; however, since Wnt pathway deregulation has been connected to CeD pathogenesis, they also hypothesise that a Wnt/PRC2 disrupted axis could cause the development of crypts hyperplasia in CeD[39] (Table 2). However, among the external factors that can influence the epigenetic regulation, the interaction with the microbiota needs also to be considered, since changes in the gut bacterial populations can affect the activity of enzymes involved in epigenetic regulation[40,41].

Table 2 Celiac disease-relevant histone modifications

Modification		Focus	Result	Relevance in CeD
H3K27ac [36]	Activation and enhancing of transcription	Profiling in stimulated CTLs from CeD subjects	IL-15, IFNβ and IL-21 induce specific acetylation profiles in CTLs	Strong association between H3K27ac and gene expression IFNβ-induced IL-15-derived changes related to lncRNAs
H3K4me3 [37]	Active transcription marker	Shared variants between CeD and RA	Similar histone enrichment in shared variants in simple cells	Different histone enrichment in disease-related specialized cells
H3K36me3 [38]	Active transcription marker	SETD2 silenced in 32% of EATL	↓H3K36me3 → γδT cells expansion	Predisposition to lymphomagenesis
H3K27me3 [39]	Gene silencing	PRC2–driven trimethylation	Villi → H3K27me3 on proliferation and differentiation genes Crypts → H3K27me3 on nutrient transport and cell killing genes	PRC2 methylation help maintenance of Wnt homeostasis → deregulation linked to CeD crypts hyperplasia

CeD: Celiac disease; CTLs: Cytotoxic T lymphocytes; LncRNAs: Long non-coding RNAs; ECM: Extracellular matrix; RA: Rheumatoid arthritis; EATL: Enteropathy associated T cell lymphoma; PRC2: Polycomb repressive complex 2.

LONG NON-CODING RNAS

Long non-coding RNAs (lncRNAs) have emerged in recent years as a class of transcripts with a wide spectrum of mechanisms of action that can affect gene expression regulation both at transcriptional and post-transcriptional level, in the nucleus and in the cytosol. Their genomic location is various, either being intergenic or within the introns of coding genes. Among their many mechanisms of action, the most frequent are direct DNA sequence interaction, transcription factor sequestration, chromatin rearrangement, regulation of histone modifications and microRNA sponging (Figure 2)[42,43]. Being implicated in so many regulatory patterns, they have been studied in cancer and in many other diseases, also with an autoimmune background[44].

The pathogenesis of CeD involves several different mechanisms, *i.e.*, the passage of the gluten peptides across the intestinal barrier, but also the activation of innate and adaptive immune response. LncRNAs can influence all these processes, but the expression of these RNAs can, in turn, be affected by the presence of SNPs.

A typical aspect in CeD pathogenesis is the loosening of the tight junctions (TJ), that increases the intestinal permeability[45]. Jauregi-Miguel *et al*[46] identified, in a genomic region associated with CeD, a lncRNA named RP4-587D13.2. This is located in an intron of *MAGI2* gene, a scaffold protein present in the tight junction plaque and able to regulate epithelial integrity. Interestingly, RP4-587D13.2 can regulate MAGI2 expression, altering the downstream TJ-related proteins like CLDN1 and ZAK. RP4-587D13.2 and MAGI2 resulted also downregulated in CeD samples (both with active CeD and on a GFD) and it seems that gliadin stimulation can reinforce this effects[46].

Ricaño-Ponce *et al*[47] identified genes located in close proximity to autoimmune-related SNPs, revealing that 42 of these SNPs could specifically affect the expression of 53 non-coding RNAs. In particular, two lncRNAs were associated to CeD-specific SNPs, namely AP002954.4 and AC104820.2. In both cases the SNPs were able to affect the expression of these lncRNAs, which had a role in the immune response[47]. Interestingly, AC104820.2 had already been detected upregulated in active CeD patients intestinal mucosa by Plaza-Izurieta *et al*[48], who also identified the SNP rs1018326 as associated with this lncRNA. These data thus support the involvement of lncRNAs in the immune response in CeD, however there are aspects that must be kept in mind, as also reported by Hrdlickova *et al*[49]. In fact, although the authors suggested the lncRNAs RP3-395 M20.9 and IL21-AS to be relevant in CeD, and linked them to TNF and IL-21 pathways, they also reported that the same lncRNAs are involved in different autoimmune disorders. In addition, some lncRNAs were detected only in some specific cell subtypes, underlying the need for a cell-specific analysis[49].

Castellanos-Rubio *et al*[50] identified lnc13 as associated to the susceptibility to celiac disease, demonstrating also the functional role of this lncRNA. Lnc13 binds the nuclear ribonucleoprotein hnRNPD and histone deacetylase 1, forming a complex that acts as a repressor of inflammatory gene expression. The presence of inflammatory stimuli activate the NFkB pathway that reduces lnc13, thus removing the repression

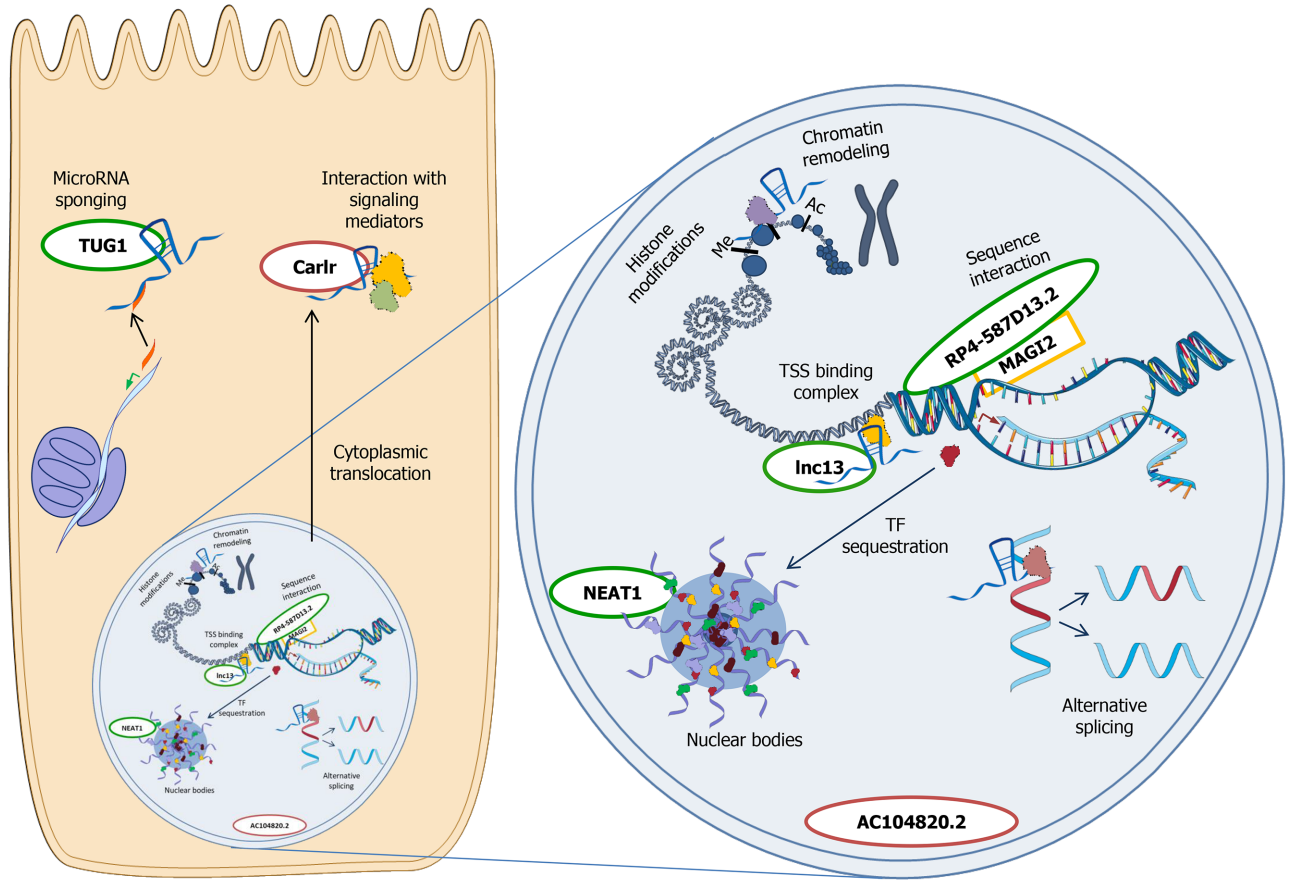

Figure 2 Main mechanisms of action of long non-coding RNAs in the cell. Cytoplasm - microRNA sponging: microRNAs are sequestered from their mRNA targets, resulting in mRNA translation; Interaction with signalling mediators: binding to pathway mediators can modulate downstream targets activation. Nucleus - chromatin remodeling: Long non-coding RNAs (lncRNAs) interact with chromatin and modify its conformation; histone modifications: lncRNAs influence the activity of the enzymes responsible for histone modifications; sequence interaction: lncRNAs act on nearby genes transcription; transcription start site (TSS) binding complex: lncRNAs bind ribonucleoproteins, interfering with gene transcription; transcription factor (TF) sequestration in nuclear bodies: TF are moved to nuclear bodies from the promoter region of target genes, influencing gene transcription. Nuclear bodies like paraspeckles consist in a lncRNA scaffold and target proteins; alternative splicing: lncRNAs determine a preferential splicing in favour of an isoform respect to another one. The so-far investigated lncRNAs in celiac disease (CeD) are reported near their known mechanism of action, respecting their main cellular localization. lncRNAs in green were reported to be downregulated in CeD, whereas the ones in red were found upregulated in a specific cell compartment. The image is original and was created with the use of Servier Medical Art modified templates, licensed under a Creative Common Attribution 3.0 Unported License (https://smart.servier.com). TSS: Transcription start site; TF: Transcription factor.

on gene transcription. Interestingly, in CeD patients, not only lnc13 expression is reduced, but there is also a SNP that generates a lnc13 variant that binds hnRNPD less effectively, possibly contributing to CeD development[50]. NFkB activation is central in CeD-driven inflammation, and it is able to induce the expression of another lncRNA, *i.e.*, Carlr. This molecule is localized in the nucleus, but it translocates in the cytosol after the activation of the NFkB pathway, and it seems essential for the induction of downstream genes such as IL-1β in macrophages. However, differently from what expected, Carlr was downregulated in total biopsies from active CeD patients, although its cytosolic fraction was increased; this observation thus needs further evaluation to determine the real role of this lncRNA in the intestinal tissue[51].

Finally, it is known that IL-15 production has a central role in CeD pathogenesis [52]. Zorro *et al*[36] in their work highlighted a subset of ncRNAs that were downregulated by IL-15 stimulation, suggesting the involvement of this class of transcripts in IL-15 response. A recent study by our group demonstrated that gliadin induces TUG1 and NEAT1 expression in biopsies obtained from CeD patients on gluten-free diet, mechanism that depends on innate immunity activation in the case of NEAT1. In fact, IL-15 can trigger NEAT1 expression both *ex vivo* and in vitro, through the newly identified axis IL-15/STAT3/NEAT1[53]. The effects of NEAT1 upregulation deserve more investigation, since this lncRNA localizes in the nucleus and is able to bind transcription factors, thus sequestering them and, in turn, regulating the expression of several genes, such as IL-8[54].

RNA METHYLATION

A further regulation can be played by RNA modifications. In fact, recent findings are pointing out that direct sequence methylation can also happen at RNA level. Differently from DNA, RNA can undergo many more kinds of methylation (up to 72), involving both mRNA and non-coding RNAs. Previous studies found RNA methylation linked to biological and immune processes, as well as complex pathologies like obesity[55]. Up to now, the main limitation in RNA methylation studies was the difficulty to perform a site-specific detection and quantification. Using a recently developed method that allows to detect m6A, the most frequent RNA modification, Olazagoitia-Garmendia *et al*[56] characterised the CeD-associated SNP rs3087898, located in the 5' untranslated region of exportin1 gene, a modulator of NFkB pathway. The mRNA derived from the CeD-associated variant was preferentially methylated with respect to the normal one, and this modification was able to increase the protein production, resulting in an inflammatory microenvironment. Besides, gliadin was able to increase this modification and exportin1 protein levels, both *in vitro* and in CeD patients samples[56]. Altogether, these studies suggest that attention should be given also to this kind of modifications when focusing on gene expression regulation in CeD.

MICRORNAS

MicroRNAs (miRNAs) are short RNA sequences (20-25 nucleotides) accounting for about 30% of gene expression regulation, affecting various processes, including chromatin conformation and transcription, as well as mRNA stability and translation. Usually they present an inverse relationship with their targets, inducing their downregulation.

miRNAs can also be detected in the plasma, and for this reason they have been evaluated in a large number of different disorders, in the attempt to identify specific biomarkers for early diagnosis or follow up. Initial papers on CeD screened the duodenal tissue to identify a specific signature representative of the events in the mucosa, and to determine whether the "signature" miRNAs were returning to normal levels in patients on GFD. Capuano *et al*[57] evaluated biopsies of children with CeD at diagnosis or on GFD, identifying about 20% of the analyzed miRNAs as differentially regulated. The authors then focused on the upregulation of miR-449a[57], which targets Notch1 and KLF4, genes involved in intestinal cell proliferation and differentiation. Indeed Notch1 expression was significantly downregulated in CeD biopsies, confirming the regulatory role of miRNAs also on proliferation in CeD. Other miRNAs were also downregulated, *i.e.*, miR-124a, miR-189, miR-299-5p and miR-379, similarly to what reported in Crohn's disease[58].

Studies on adult CeD patients identified different subset of miRNAs according to the clinical picture or the severity of intestinal damage[12,14]. A significant downregulation of miR-31-5p was observed in all the CeD groups, whereas miR-192-3p and miR-192-5p were downregulated in CeD patients with anemia and a severe histological lesion, respectively. miR-192-5p targeted two different molecules involved in the innate immunity, *i.e.*, NOD2 and CXCL2, that were upregulated in CeD patients, in particular in severe cases[12]. On the other hand, miR-31-5p had as a target Foxp3, essential for Treg development; again, a significant inverse correlation was observed between the miRNA and the target mRNA. These changes were induced by gliadin, as demonstrated by *in vitro* stimulation of biopsies of patients on GFD[12]. It is interesting to note that the data on miR-192 are similar to those observed in IBDs, in which miR-192 regulated NOD2 expression[59].

However, a more comprehensive approach was needed, and a recent paper correlated, in the duodenal mucosa of celiac subjects, the miRNA and mRNA expression patterns obtained by sequencing[17]. The data analysis revealed the presence of a complex network involving various pathways known to be deregulated in CeD, such as immunity (interferon), suggesting that CeD-associated miRNAs play a central role in causing the intestinal damage.

The identification of a specific miRNA plasma signature remains elusive at the present time. An analysis performed in pediatric CeDs revealed the presence of a trend similar to that observed in duodenal biopsies for miR-192-5p, miR-31-5p and miR-21-5p. However none of these miRNAs could be defined as a marker able to evaluate the recovery of the mucosa on GFD, since there was no return to normal levels (miR192-5p) or a clear cut-off could not be established (miR-31-5p and miR-21-5p)[13]. A

further study performed by Amr *et al*[60] analyzed the same miRNAs, *i.e.*, miR-21 and miR-31, identifying a cutoff value and determining a sensitivity and specificity of 82.4%and 80.8% for miR-21 and 93.8% and 72% for miR-31. Another recent paper confirmed the value of plasmatic miR-21 as a marker, with a sensitivity and specificity of 0.65 and 0.83, but better results were obtained considering miR-155 (0.94 and 0.87, respectively)[61]. Although encouraging, these results need further validation in larger cohorts; moreover even for miR-21 the identified cutoff values were quite dissimilar, thus suggesting the need to determine the cutoff according to the method and equipment employed for the analysis. Last but not least, the data obtained in CeD patients will have to be compared with those obtained from subjects with other inflammatory disorders such as IBDs, to confirm the specificity of the findings.

MACHINE LEARNING

As described, the evaluation of the epigenome generates large datasets, and the data increases when the integration of different datasets (such as those derived from methylome and long/short non-coding RNA transcriptome) is required. The generation of a large quantity of data allows to move towards a data-driven analysis, *i.e.*, machine learning or artificial intelligence (these terms are now regarded as interchangeable). Machine learning can, through statistical methods and algorithms, make classifications or predictions of input data, such as recognize a specific endoscopy pattern and classify the patient as celiac or non-celiac. In simpler machine learning approaches supervised learning is commonly used, *i.e.*, the use of labeled datasets designed to train or "supervise" algorithms into classifying data or predicting outcomes. In the medical field this obviously requires training datasets selected by specialized clinicians (for example the identification of endoscopic images characteristic of celiac disease). Deep learning is a subset of machine learning, in which the algorithms are more complex and include more than one layer in the neuronal network. For deep learning the presence of a labeled training dataset is not required, since these algorithms can analyze data in the raw form and determine the features that distinguish the various groups.

With regards to CeD, the machine learning approach has been originally used to analyze endoscopic data, either those generated by upper endoscopy[62] or by videocapsule and, in this latter case, the deep learning approach was able to reach a high sensitivity and specificity in the diagnosis of CeD using the video capsule images [63,64]. Although these data seem quite encouraging, it must be kept in mind that the gastrointestinal tract is a complex environment, and several factors can affect the obtained images, as discussed in depth by Hegenbart *et al*[65]. The deep learning approach could be useful also in the evaluation of duodenal biopsies, as demonstrated by different authors that were able to develop artificial intelligence-based methods for the correct classification of duodenal samples[66-68]. Machine learning has also been employed to improve the diagnosis based on B/T cell repertoire[69,70]; although the data obtained in the latter case are quite interesting, this approach is not easily applicable for routine diagnosis.

A machine learning approach could have a very important clinical application should it be able to predict who, within a risk population, will develop the disease. An encouraging study that analyzes this aspect has recently been published by Piccialli *et al*[71], who tested different models in order to predict the development of an overt CeD in childen with potential CeD. The use of a machine learning approach towards basic science data obtained from duodenal biopsies of CeD patients has recently been published by Wolf *et al*[16], who analyzed not only the trascriptomic profiles but also the expression of some lncRNAs and miRNAs. Moreover, the authors focused on gene expression signatures which associate with transcription factor (TF)-activity and chromatin state, as well as DNA and histone methylation pattern, thus providing an indirect measure of epigenetic modifications. This comprehensive analysis revealed, for example, a different role of miRNAs and lncRNAs in the regulation of gene expression, correlating the latter category with more subtle adjustments of the transcription under control of epigenetic mechanisms.

CONCLUSION

Understanding the epigenetics of celiac disease remains mandatory in order to completely clarify the pathogenetic processes behind its development and the

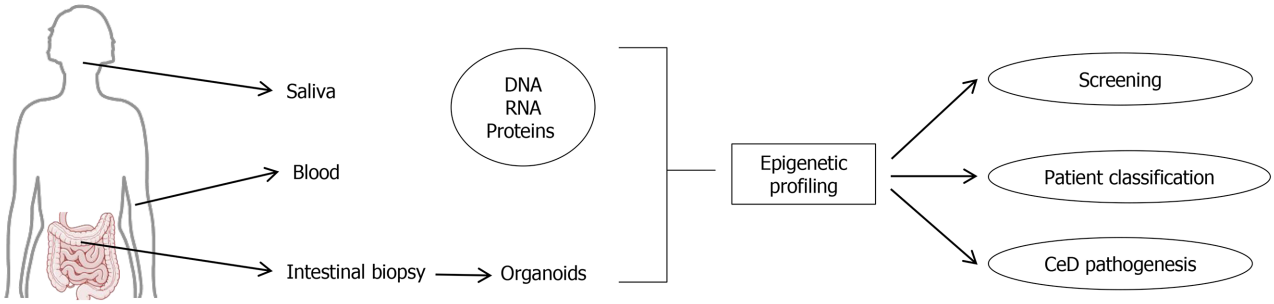

Figure 3 Potential use of epigenetics studies in celiac disease. The image is original and was created with the use of Servier Medical Art modified templates, licensed under a Creative Common Attribution 3.0 Unported License (https://smart.servier.com). CeD: Celiac disease.

spectrum of its manifestations. The data here reviewed demonstrate that epigenetic changes occur in celiac disease, however further data are still needed before the identification of these changes could be used to screen or better categorize the patients, as depicted in Figure 3. In particular it will be essential to verify that the characteristics detected at a peripheral level (*i.e.*, blood or saliva) correspond to those present in the intestinal mucosa. In this regard, data seem encouraging for miRNAs, but the identification of a specific signature will probably require the combination of more than one of them to provide an excellent sensitivity and specificity.

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ORIGINAL ARTICLE

Basic Study

Knockdown of DEAD-box 51 inhibits tumor growth of esophageal squamous cell carcinoma *via* the PI3K/AKT pathway

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Abstract

BACKGROUND

Esophageal squamous cell carcinoma (ESCC) is one of the most prevalent malignancies that seriously threaten people's health worldwide. DEAD-box helicase 51 (DDX51) is a member of the DEAD-box (DDX) RNA helicase family, and drives or inhibits tumor progression in multiple cancer types.

AIM

To determine whether DDX51 affects the biological behavior of ESCC.

METHODS

The expression of DDX51 in ESCC tumor tissues and adjacent normal tissues was detected by Immunohistochemistry (IHC) analyses and quantitative PCR (qPCR). We knocked down DDX51 in ESCC cell lines by using a small interfering RNA (siRNA) transfection. The proliferation, apoptosis, and mobility of DDX51 siRNA-transfected cells were detected. The effect of DDX51 on the phosphoinositide 3-kinase (PI3K)/AKT pathway was investigated by western blot analysis. A mouse xenograft model was established to investigate the effects of DDX51 knockdown on ESCC tumor growth.

RESULTS

DDX51 exhibited high expression in ESCC tissues compared with normal tissues and represented a poor prognosis in patients with ESCC. Knockdown of DDX51 induced inhibition of ESCC cell proliferation and promoted apoptosis. Moreover, DDX51 siRNA-expressing cells also exhibited lower migration and invasion rates. Investigations into the underlying mechanisms suggested that DDX51 knock-

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down induced inactivation of the PI3K/AKT pathway, including decreased phosphorylation levels of phosphate and tensin homolog, PI3K, AKT, and mammalian target of rapamycin. Rescue experiments demonstrated that the AKT activator insulin-like growth factor 1 could reverse the inhibitory effects of DDX51 on ESCC malignant development. Finally, we injected DDX51 siRNA-transfected TE-1 cells into an animal model, which resulted in slower tumor growth.

CONCLUSION

Our study suggests for the first time that DDX51 promotes cancer cell proliferation by regulating the PI3K/AKT pathway; thus, DDX51 might be a therapeutic target for ESCC.

Key Words: Esophageal squamous cell carcinoma; DDX51; PI3K/AKT pathway; Tumor growth; Therapeutic target

Core Tip: Our study revealed that DEAD-box helicase 51 was upregulated in esophageal squamous cell carcinoma (ESCC) tumor tissues and promoted tumor proliferation and development by upregulating the phosphorylation of phosphoinositide 3-kinase/AKT pathway members. These data extend our knowledge of the function and molecular mechanism of the DEAD-box family in tumor biology and provide a potential therapeutic target for ESCC treatment.

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is the sixth leading cause of cancer-related deaths, causing approximately 300,000 deaths each year[1-3]. China has the highest incidence of ESCC globally, caused by multiple factors such as diet and the environment. At present, surgical treatment is the most effective means to cure ESCC. However, surgical treatment is highly challenging due to the close distance between the esophagus and life-supporting structures such as the respiratory system[4]. Chemotherapy and radiation therapy are commonly used as adjuvant treatments. The main problem with these two treatments is inherent resistance, and even local ESCC is often resistant[5,6]. Due to the extremely high tumor recurrence rate (about 40%) and frequent local lymph node metastases, the prognosis of ESCC patients is very poor, with a 5-year survival rate of only 10%-30%[1]. Molecular targeted therapy has shown good application prospects in ESCC patients in recent years. To improve the survival of ESCC patients, it is essential to explore effective molecular targets for ESCC treatment.

DEAD-box (DDX) RNA helicases, belonging to helicase superfamily 2, comprise the largest group of RNA helicases[7]. There are at least 31 members of DDX family in humans, including DEAD-box helicase 51 (DDX51). DDX proteins are highly conserved helicases that catalyze duplex unwinding and structural remodeling of RNA or ribonucleoprotein complexes[8]. They play important roles in nearly all aspects of RNA metabolism including RNA transcription, post-transcriptional splicing, ribosome assembly, and RNA degradation[9]. In recent years, DDX family members have been identified to be dysregulated and function as tumor oncogenes or suppressors in various tumor types[10]. For example, knockdown of DDX5 exerts inhibitory effects on the proliferation and epithelial-to-mesenchymal transition of ESCC cells by downregulating β-catenin, c-Myc, and cyclin D1[11]. DDX51 is a recently identified member of the DDX family. DDX51 is mainly responsible for maturation of the 3′ end of 28S and its proliferation-promoting activities have been

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demonstrated in non-small cell lung cancer (NSCLC) and breast cancer[12,13]. However, whether DDX51 plays a role in the progression of ESCC is still unclear.

In this study, we investigated the function of DDX51 in ESCC cell proliferation and tumor growth by using a small interfering RNA (siRNA) silencing approach.

MATERIALS AND METHODS

Tumor specimen collection and immunohistochemistry analysis

ESCC tumor tissues and adjacent normal tissues (n = 118) were obtained from patients with ESCC between 2016 to 2020 in Shandong Provincial Hospital Affiliated to Shandong First Medical University (Shandong, China). The Research Ethics Committee approved all experimental procedures of Shandong Provincial Hospital Affiliated to Shandong First Medical University. Patients or their guardians wrote the informed consents. The clinicopathological parameters of ESCC patients were collected. Immunohistochemistry (IHC) staining was performed to investigate the expression of DDX51 in ESCC tissues and adjacent normal tissues, and the experimental procedure is described in a previous report[14].

Cell culture and transfection

The ESCC cell lines Eca109 and TE-1 were purchased from the Cell Bank of the Chinese Academy. The cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin in a 37 °C cell incubator. When cells were grown to 70% confluence, si-DDX51 or negative control siRNA (si-NC) was transfected into Eca109 and TE-1 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, United States) according to the corresponding experimental groups. The stable DDX51 knockdown ESCC cell line was established by using lentivirus transfection. Lentivirus expressing si-DDX51 or si-NC was purchased from Genechem (Shanghai, China). After 48 h of transfection, cells were collected and analyzed for DDX51 expression using quantitative PCR (qPCR) and western blot analysis.

RNA extraction and qPCR

Total RNA was extracted from ESCC tumor tissues and cell lines using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, United States). Then 1 μg total RNA was reverse-transcribed to cDNA using the PrimeScript RT Master Mix (Takara Biotechnology Co., Ltd., Dalian, China). The expression of DDX51 was determined *via* qPCR with the FastStart Universal SYBR Green Master (Roche, Germany). The experiment was performed on the C1000 Thermal Cycler (Bio-Rad, Hercules, CA, United States). The PCR reaction conditions were as follows: Pre-degeneration at 95 °C for 5 min, followed by 45 cycles of 95 °C for 5 s, 60 °C for 10s, and 72 °C for 10 s. GAPDH was used as an internal control.

Cell Counting Kit-8 assay

After transfection, Eca109 and TE-1 cells were seeded into 96-well plates at a density of 3000 cells per well. Each group had three wells. The Cell Counting Kit-8 (CCK-8; Dojindo, Kyushu, Japan) was used to detect the cell viability of transfected cells. At specific time points (24, 48, 72, and 96 h), 10 μL CCK-8 reagent was added to each well and incubated at 37 °C for 2 h. Cell viability was represented by the absorbance at 450 nm, which was detected on a spectrophotometer (Molecular Devices, San Jose, CA, United States).

Flow cytometry

Cell apoptosis was detected by using the Annexin V-FITC/Propidium Iodide (PI) Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, United States) following the manufacturer's instructions. The transfected cells were digested with 0.25% EDTA-free trypsin and resuspended in binding buffer. Then 100 μL cell suspension was incubated with 5 μL AnnexinV-FITC and 5 μL PI for 30 min. The incubation was performed at 37 °C in the dark. The apoptosis percentage was analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, United States).

Scratch assay

A scratch assay evaluated the effect of cell transfection on its migration ability. Eca109 and TE-1 cells were seeded in a 6-well plate at a density of 1 × 10^5 cells/mL. Then cells

were transfected as described as before. After 16 h, cell transfection was stopped and cells were continuously cultured to 100% confluence. Then a single-line scratch was created on the cell monolayer using a sterile micropipette tip. Phosphate-buffered saline was used to wash the cell debris, and serum-free medium was added to the plates. Cells were photographed at 0 and 24 h under an inverted fluorescence microscope (IX-71). The width of the scratch was measured. All experiments were performed in triplicate and repeated at least three times.

Transwell invasion assay

Cell invasion was detected using a transwell assay. Transwell inserts (6.5 mm, 8 μm pore size; CoStar Group, Washington, DC, United States) were added to the 24-well transwell plates. The inserts were coated with 50 μL of 1 mg/mL Matrigel matrix (BD Biosciences). Eca109 and TE-1 cells were transfected as described above and the cell density was adjusted to 10^6 cells/mL. Then 200 μL cell suspension was added to the upper chamber of the transwell inserts, while 600 μL medium with 10% FBS was added to the lower well. Cells were cultured at 37 °C for 24 h, followed by fixation and staining with 1% crystal violet. The invasive cells were observed under a light microscope, and five random fields were selected to take pictures.

Western blot

Total proteins from transfected ESCC cells were extracted using radioimmunoprecipitation assay buffer with a 1% protease inhibitor. The protein concentration in the extraction solution was determined using the bicinchoninic acid assay. Then 20 μg total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and proteins were electrotransferred to a PVDF membrane. The membrane was blocked in 5% bovine serum albumin containing Tris-buffered saline with Tween 20 (TBST) (10 mmol/L Tris–HCl, pH 7.4, 150 mmol/L NaCl, 0.05% Tween-20) at room temperature for 2 h, and then incubated with primary antibodies at 4 °C overnight. The primary antibodies included phosphatase and tensin homolog (PTEN) (1:1000, ab267787; Abcam, Cambridge, MA, United States), phosphoinositide 3-kinase (PI3K) (1:1000, CY5355; Abways, Shanghai, P.R. China), phosphorylated PI3K (p-PI3K) (1:1000, CY6427; Abways), AKT (1:1000, AF6261; Affinity Biosciences, Cincinnati, OH, United States), p-AKT (1:1000, AF016; Affinity Biosciences), mammalian target of rapamycin (mTOR) (1:1000, ab134903), p-mTOR (1:1000, ab137133; Abcam), and anti-GAPDH (1:5,000; ab8245; Abcam). After washing with TBST buffer four times every 5 min, the membrane was incubated with secondary antibodies (Santa Cruz, CA, United States) for 1 h at room temperature and washed with TBST another four times. Finally, proteins were visualized by chemiluminescence (Santa Cruz Biotechnology, Dallas, TX, United States).

In vivo tumorigenesis assay

Male nude mice (6-wk-old) were obtained from Shanghai SIPPR-BK Laboratory Animal Co. Ltd. (Shanghai, P.R. China). All animal experiments were approved by the Shandong Provincial Hospital Affiliated to Shandong First Medical University. The animals were maintained in specific pathogen-free conditions in accordance with the institutional animal care and use committee regulations. ESCC cells were transfected with lentivirus-NC or lentivirus-si-DDX51 for 48 h and then resuspended in a serum-free medium. The cell density was adjusted to 2×10^7/mL. Each mouse was subcutaneously injected with 5×10^6 of ESCC cells. The tumor length and width were monitored every other day and length × width^2/2 represented tumor volume. On day 28, nude mice were sacrificed by carbon dioxide asphyxiation.

Statistical analyses

All experimental data are expressed as the mean ± standard deviation from three independent experiments. The difference between the two groups was analyzed using the Student's *t*-test. The difference among multiple groups was evaluated using one-way analysis of variance. $P < 0.05$ was considered statistically significant. GraphPad Prism 5 Software (GraphPad, San Diego, CA, United States) was used for all statistical analyses. All data in this study satisfied parametric test assumptions.

RESULTS

DDX51 is highly expressed in ESCC tumor tissues and is associated with a poor prognosis in ESCC patients

DDX51 is upregulated in breast cancer. Here, we detected the expression of DDX51 in ESCC tissues, which might provide insights into its role in ESCC. As shown in Figure 1A, we performed IHC analysis of DDX51 expression in the tumor tissues and adjacent normal tissues of patients with ESCC. The results showed strong IHC staining of ESCC tissues, which was very weak in the normal controls. Meanwhile, DDX51 expression was detected by qPCR, which also showed significantly higher expression in ESCC tumor tissues than in normal adjacent tissues (Figure 1B). Furthermore, the correlation between DDX51 expression with patients' clinicopathological features was analyzed. As shown in Table 1, DDX51 expression did not correlate with patients' age, sex, tumor size, or T stage, but was associated with tumor differentiation degree, N stage and tumor-node-metastasis (TNM) stage. Survival analysis results indicated that ESCC patients with high expression of DDX51 had lower survival rates compared to those with low expression of DDX51 (Figure 1C). These results suggest that DDX51 exhibits high expression in ESCC tumor tissues and may be associated with a poor prognosis in patients with ESCC (Table 2).

Knockdown of DDX51 plays an anti-tumor role in ESCC cells

To investigate the function of DDX51 in ESCC progression, we performed a knockdown experiment in Eca109 and TE-1 cells using siRNA transfection. The transfection efficiencies are shown in Figure 2A-C. The qPCR results indicated that, compared with the NC group, the mRNA expression of DDX51 in the si-DDX51 group was decreased to 44.5% in Eca109 cells and 18.8% in TE-1 cells (Figure 2A and B, both $^{a}P < 0.05$). Western blot analysis showed that si-DDX51 transfection led to a decrease in DDX51 protein expression to 49.2% in Eca109 cells and 52.7% in TE-1 cells (Figure 2C, both $^{a}P < 0.05$), demonstrating the effective knockdown of DDX51 in both cell lines. Next, we investigated the effects of DDX51 knockdown on the malignant progression of ESCC cells. Cell viability and flow cytometry results suggested that the proliferation rates of Eca109 and TE-1 cells were significantly inhibited by DDX51 knockdown (Figure 2D and E), with a significant increase in cell apoptotic rates (Figure 3A and B). Furthermore, the wound healing rates and invasive cell number were significantly decreased in the si-DDX51 group compared with the NC group (Figure 3C-F). Taken together, these data show that DDX51 functions as an oncogene in ESCC progression.

The anti-tumor effects of DDX51 knockdown on ESCC are partly mediated by the PI3K/AKT signaling pathway

The PI3K/AKT signaling pathway is constitutively activated in multiple cancer types and extensively participates in tumor cell proliferation, apoptosis, metastasis, and other malignant phenotypes. Here, we determined whether the oncogenic function of DDX51 in ESCC involves the PI3K/AKT signaling pathway. As shown in Figure 4A-E, the expression of members in the PI3K/AKT signaling pathway including PTEN, p-PTEN, PI3K, p-PI3K, AKT, p-AKT, mTOR, and p-mTOR was evaluated by western blotting. The results showed that DDX51 knockdown induced a significant decrease in the phosphorylation levels of PTEN, PI3K, AKT, and mTOR, while the total protein was not affected, suggesting that the PI3K/AKT signaling pathway was inactivated in DDX51-knockdown ESCC cells. To further elucidate the role of the PI3K/AKT signaling pathway in the function of DDX51, we applied an AKT agonist insulin-like growth factor 1 (IGF-1), which induced persistent activation of the AKT pathway. IGF-1 treatment reversed the anti-proliferation and pro-apoptotic effects of DDX51 knockdown in ESCC cells (Figure 4F and G). Meanwhile, the migration and invasion abilities were also increased to the NC level when ESCC cells were treated with both si-DDX51 and IGF-1 (Figure 5). These results suggest that the anti-tumor effects of DDX51 knockdown in ESCC are, at least in part, mediated by the PI3K/AKT signaling pathway.

Knockdown of DDX51 slows down the growth of ESCC cells

We further investigated the effects of DDX51 knockdown on ESCC tumor growth *in vivo*. TE-1 cells were transfected with lv-si-DDX51 or lv-NC for 48 h and subcutaneously injected into the right flanks of nude mice. Six repeats were designed for each group. The tumor volume was measured and analyzed every other day. At the end of the experiment, tumor weights were measured and DDX51 expression was

Table 1 Correlation of DDX51 expression with the clinicopathologic features of ESCC patients

Characteristics	Cases (*n* = 118)	DDX51 expression level		*P* value
		Low (*n* = 52)	High (*n* = 66)	
Age (yr)				0.740
< 50	30	14	16	
≥ 50	88	38	50	
Gender				0.201
Male	91	43	48	
Female	27	9	18	
Tumor size				0.194
< 30 mm	20	12	8	
30-50 mm	66	29	37	
≥ 50 mm	32	11	21	
Differentiation degree				0.025[a]
High	26	11	15	
Moderate	53	30	23	
Low	39	11	28	
pT				0.154
T1	8	6	2	
T2	51	23	28	
T3 + T4	59	23	36	
pN				0.012[a]
N0	42	25	17	
N1 + N2	76	27	49	
pTNM				0.002[a]
I + II	37	24	13	
III + IV	81	28	53	

[a]*P* < 0.05 indicated statistical significance. Statistical analysis was performed using the chi-square test. TNM: Tumor-node-metastasis.

Table 2 Comparison of Kaplan–Meier survival curves

	B	SE	Wald	*P* value	HR	95%CI
Differentiation	0.052	0.157	0.110	0.740	1.053	0.774-1.433
pN	0.029	0.451	0.004	0.949	1.029	0.425-2.491
pTNM	1.078	0.518	4.323	0.038	2.939	1.064-8.119
DDX51	0.893	0.241	13.686	< 0.01	2.443	1.522-3.921

analyzed by IHC analysis. The results are shown in Figure 6, which indicated that the tumor volume of the lv-si-DDX51 group (1639.35 ± 674.36 mm^3) was significantly decreased compared with that of the NC group (1013.03 ± 264.51 mm^3) on day 28. The weight of the NC group (0.442 ± 0.116 g) was also significantly higher than that of the lv-si-DDX51 group (0.715 ± 0.156 g). These results showed that DDX51 knockdown inhibited ESCC tumor growth in the mouse xenograft model.

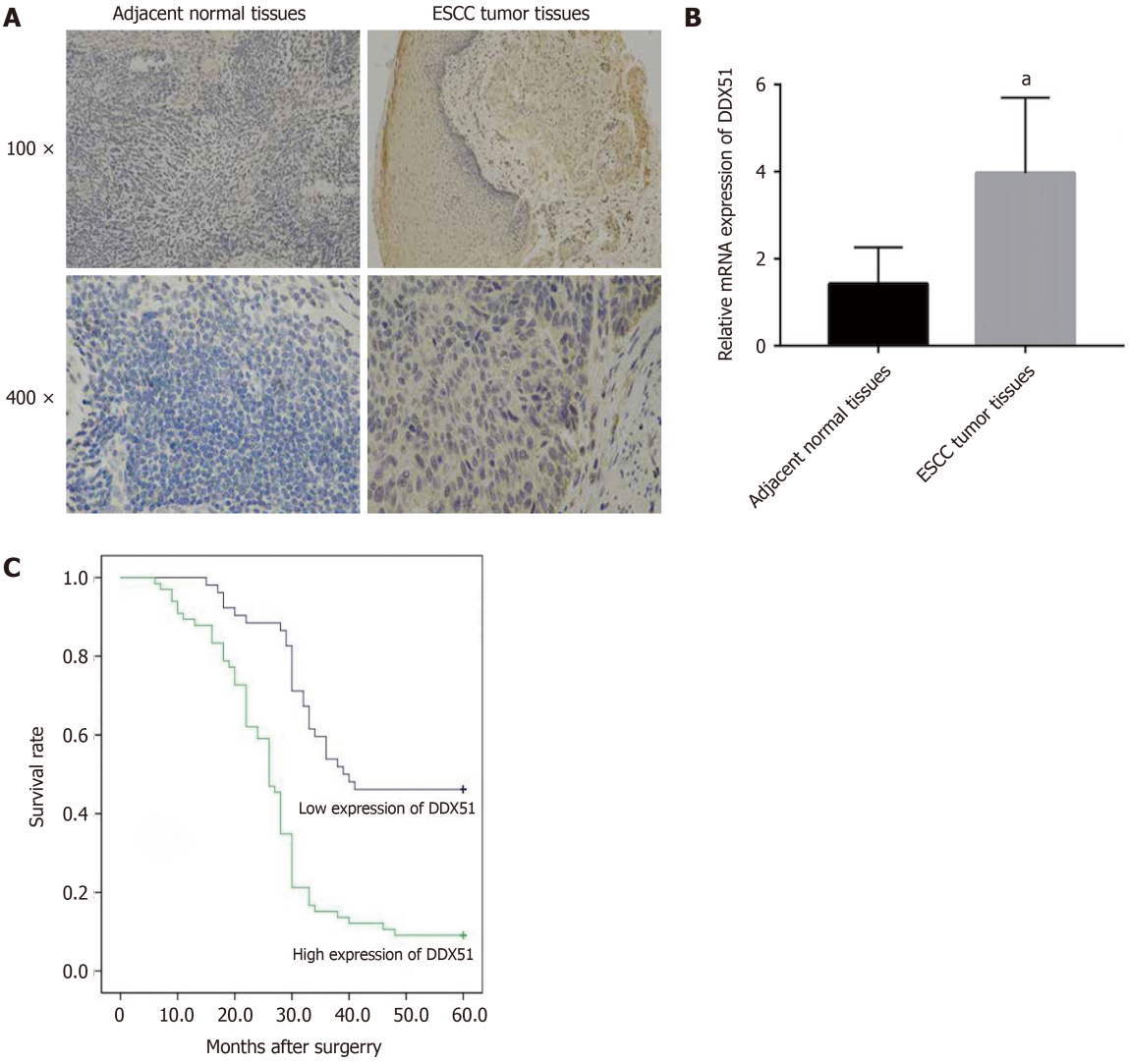


Figure 1 Expression and prognostic value of DEAD-box helicase 51 in esophageal cancer. A: Immunohistochemistry analysis of DEAD-box helicase 51 (DDX51) expression in tumor tissues and paired normal tissues of patients with esophageal squamous cell carcinoma (ESCC); B: Quantitative PCR detection of DDX51 mRNA expression in the tumor tissues (n = 118) and paired normal tissues (n = 118) of patients with ESCC, $^{a}P < 0.05$; C: Survival curves of ESCC patients with high or low expression (P = 0.0036).

DISCUSSION

The DDX family, which has activities of RNA unwinding and structure remodeling, regulates nearly all aspects of RNA-related biology, from synthesis to degradation [10]. Since mRNA serves as an intermediate between DNA genetic information and proteins, the DDX family plays crucial roles in multiple cellular processes such as cell proliferation, apoptosis, and malignant transformation[15-19]. DDX proteins ordinarily exert their function by forming a large multi-protein complex. Therefore, their exact function is probably impacted by their interacting partners and is profoundly context dependent[20,21]. The most important characteristic of a tumor is heterogeneity, which might explain the diverse functions of DDX proteins in tumors, as an oncogene or tumor suppressor[22,23]. DDX51 is a recently identified member of DDX family, which shows pro-proliferation activity in the progression of NSCLC and breast cancer[12,13].

Here we demonstrated that DDX51 was upregulated in ESCC tumor tissues and promoted tumor proliferation and metastasis. The results also suggested that the oncogenic function of DDX51 in ESCC was a result of activation of the PI3K/AKT signaling pathway, which is consistent with previous reports that revealed the regulatory action of other DDX proteins on the AKT pathway.

DDX family members are dysregulated in various tumor types, including ESCC [10]. For example, gene microarray data of breast cancer showed that DDX1 upregulation is associated with tumor occurrence and early recurrence and serves as an

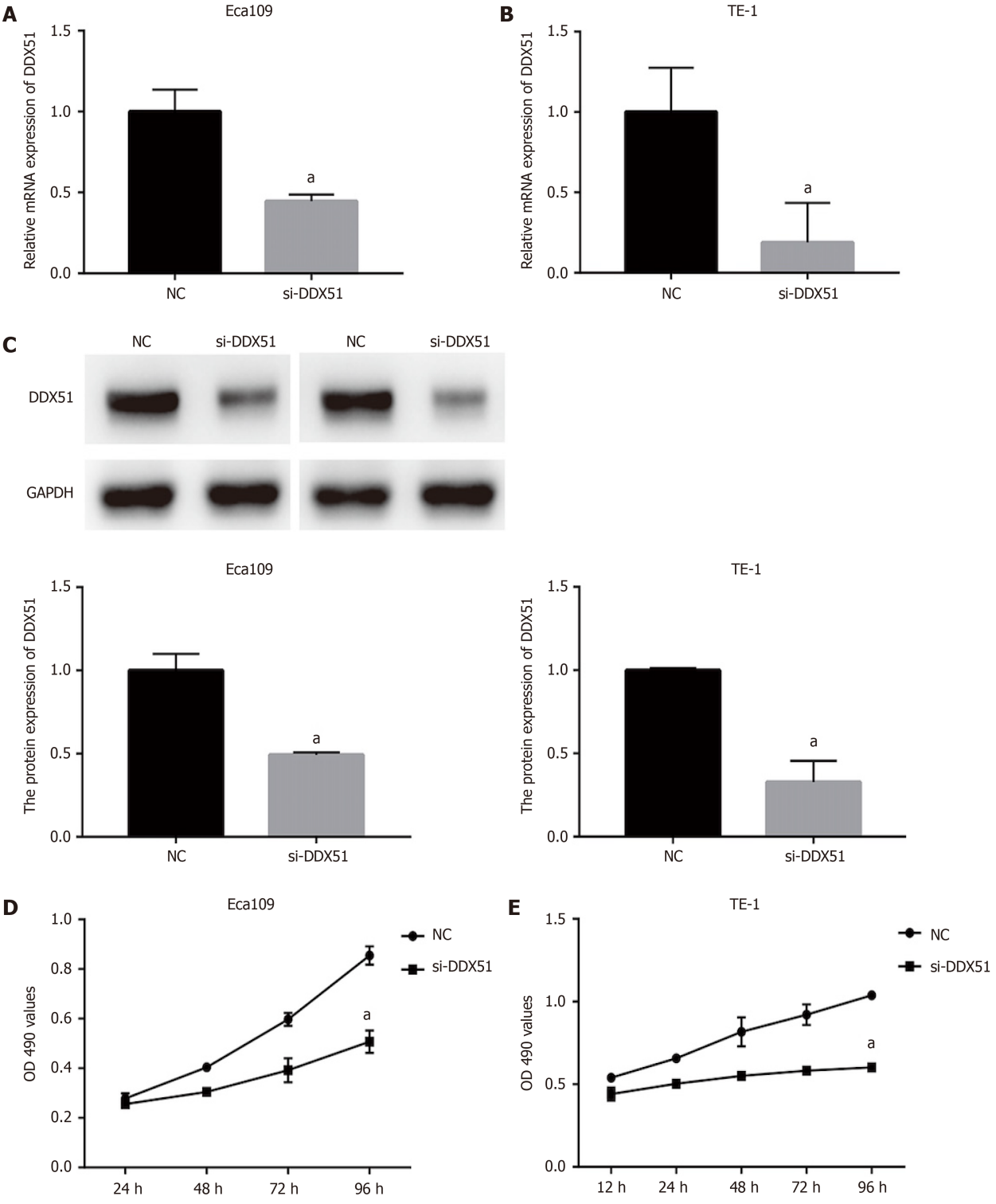
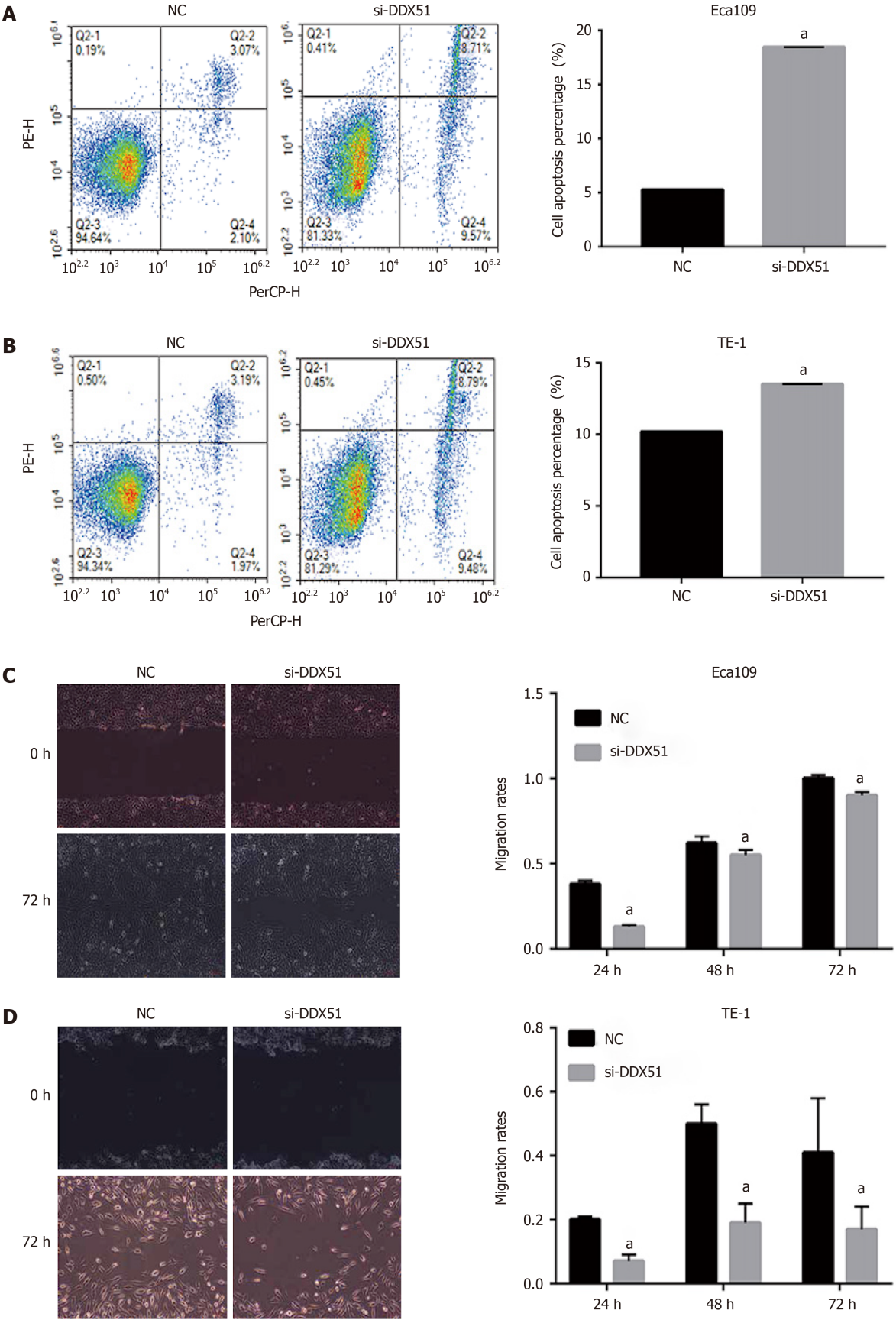


Figure 2 Anti-proliferation effects of DEAD-box helicase 51 in esophageal cancer. Negative control (NC) small interfering RNA (siRNA) and DEAD-box helicase 51 (DDX51) siRNA were synthesized and transfected into Eca109 and TE-1 cells. A and B: Quantitative PCR assay was used for the detection of knockdown efficiencies; C: Western blot analysis of DDX51 expression in DDX51 siRNA-expressing cells; D and E: The effects of DDX51 on the viability of esophageal squamous cell carcinoma cells. $^{a}P < 0.05$. All data were obtained from at least three independent experiments. NC was a scrambled siRNA.

independent prognostic biomarker for patients' survival[24]. Wang *et al*[12] investigated the expression of DDX51 in NSCLC patients. The results showed that DDX51 expression was associated with patient age but no other risk factors. In breast cancer, DDX51 also exhibits high expression in tumor tissues and predicts a high TNM stage and poor prognosis in patients[13]. Here, our results showed that DDX51 was upregulated in ESCC tumor tissues and was negatively correlated with patient survival, which was consistent with previous studies on DDX51 in tumors. Our study identified that the upregulated expression of DDX51 in ESCC is associated with the


A
NC
si-DDX51
Q2-1 0.19%
Q2-2 3.07%
Q2-3 94.64%
Q2-4 2.10%
Q2-1 0.41%
Q2-2 8.71%
Q2-3 81.33%
Q2-4 9.57%
PE-H
PerCP-H
Eca109
Cell apoptosis percentage (%)
a
NC
si-DDX51
B
NC
si-DDX51
Q2-1 0.50%
Q2-2 3.19%
Q2-3 94.34%
Q2-4 1.97%
Q2-1 0.45%
Q2-2 8.79%
Q2-3 81.29%
Q2-4 9.48%
PE-H
PerCP-H
TE-1
Cell apoptosis percentage (%)
a
NC
si-DDX51
C
NC
si-DDX51
0 h
72 h
Eca109
Migration rates
NC
si-DDX51
a
24 h
48 h
72 h
D
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si-DDX51
0 h
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TE-1
Migration rates
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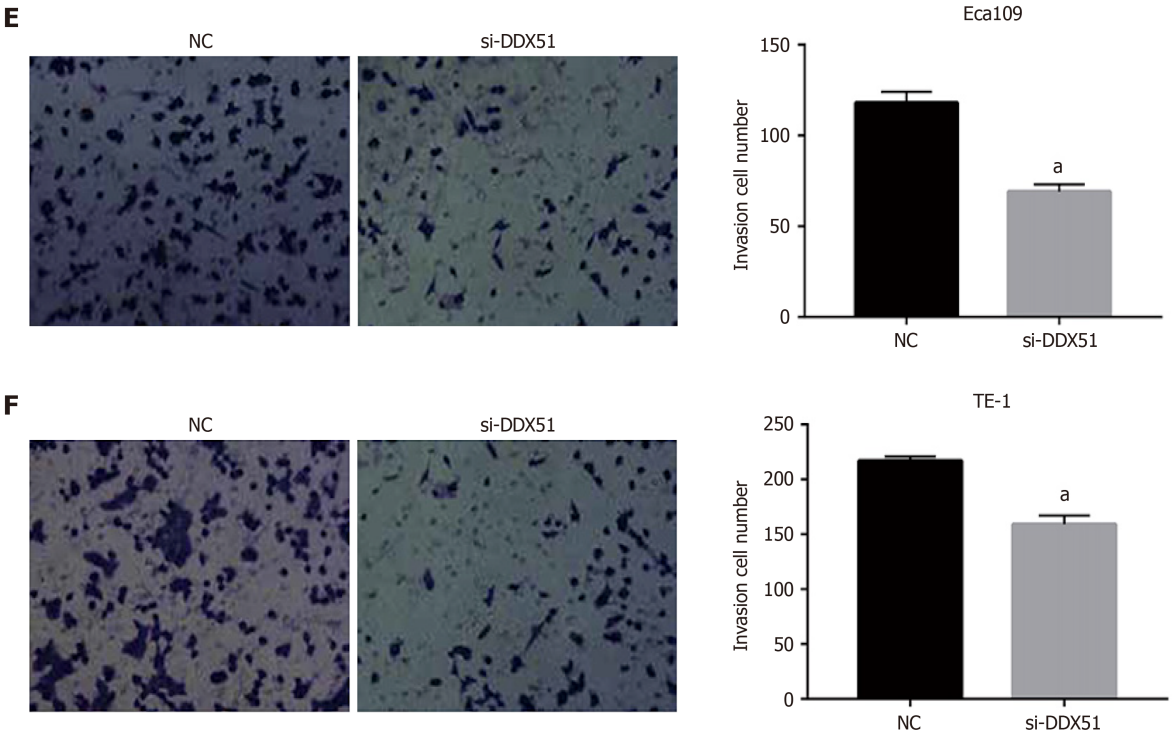

Figure 3 Effects of DEAD-box helicase 51 small interfering RNA on cell apoptosis, migration, and invasion of esophageal squamous cell carcinoma cells. A and B: Cell apoptosis percentage of DEAD-box helicase 51 (DDX51) small interfering RNA (siRNA)-expressing esophageal squamous cell carcinoma (ESCC) cells; C and D: Scratch assays for cell migration analysis of the DDX51 siRNA-expressing ESCC cells; E and F: Transwell assays for cell invasion analysis of DDX51 siRNA-expressing ESCC cells. $^{a}P < 0.05$. All data were obtained from at least three independent experiments. Negative control was a scrambled siRNA.

degree of differentiation, pathological N stage, and pathological TNM stage. The above findings suggest that DDX51 overexpression may serve as an independent prognostic factor in ESCC. However, it is currently unclear if this is an indication of the severity of the cancer itself. Further study should be performed to investigate the prognostic value of DDX51 in a larger number of specimens.

As RNA synthesis and decay involve the regulation of broad-spectrum protein expression, it is not surprising that the dysregulation of DDX proteins in tumors affects different signaling pathways. In a review reported in 2021, the signaling regulation network of DDX family in tumorigenesis was summarized including Wnt/β-catenin pathway, Snail/E-cadherin pathway, hypoxia inducible factor 1 alpha/DDX3/E-cadherin and so on[10]. For DDX51, microarray analyses revealed that DDX51 siRNA-expressing cells expressed higher levels of transforming growth factor beta receptor, interleukin 1 receptor, and c-FOS in NSCLC[12]. The Wnt/β-catenin pathway is also downregulated by DDX51 siRNA in breast cancer[13]. Here, we found that DDX51 siRNA resulted in the inactivation of the PI3K/AKT pathway, including decreased phosphorylation levels of PTEN, PI3K, AKT, and mTOR. The PI3K/AKT pathway is constitutively activated in many tumor types including ESCC, and participates in tumor cell proliferation, apoptosis, and progression[25-27]. In a previous study, DDX5 was demonstrated to occupy the AKT promoter with β-catenin as well as nuclear factor kappa B, and induces the mRNA and protein expression of AKT[28]. However, in gastric cancer miR-5590-3p targets DDX5, which further decreases the phosphorylation levels of AKT and does not affect AKT protein expression[29]. These results suggest that DDX proteins can regulate the AKT pathway through either protein expression or phosphorylation level. In the future, we intend to investigate the effects of DDX51 on transcriptome and proteome expression in tumor progression, which will provide more information on the mechanism of action of this protein.

CONCLUSION

This study had some limitations. First, *in vivo* experiments were not performed to

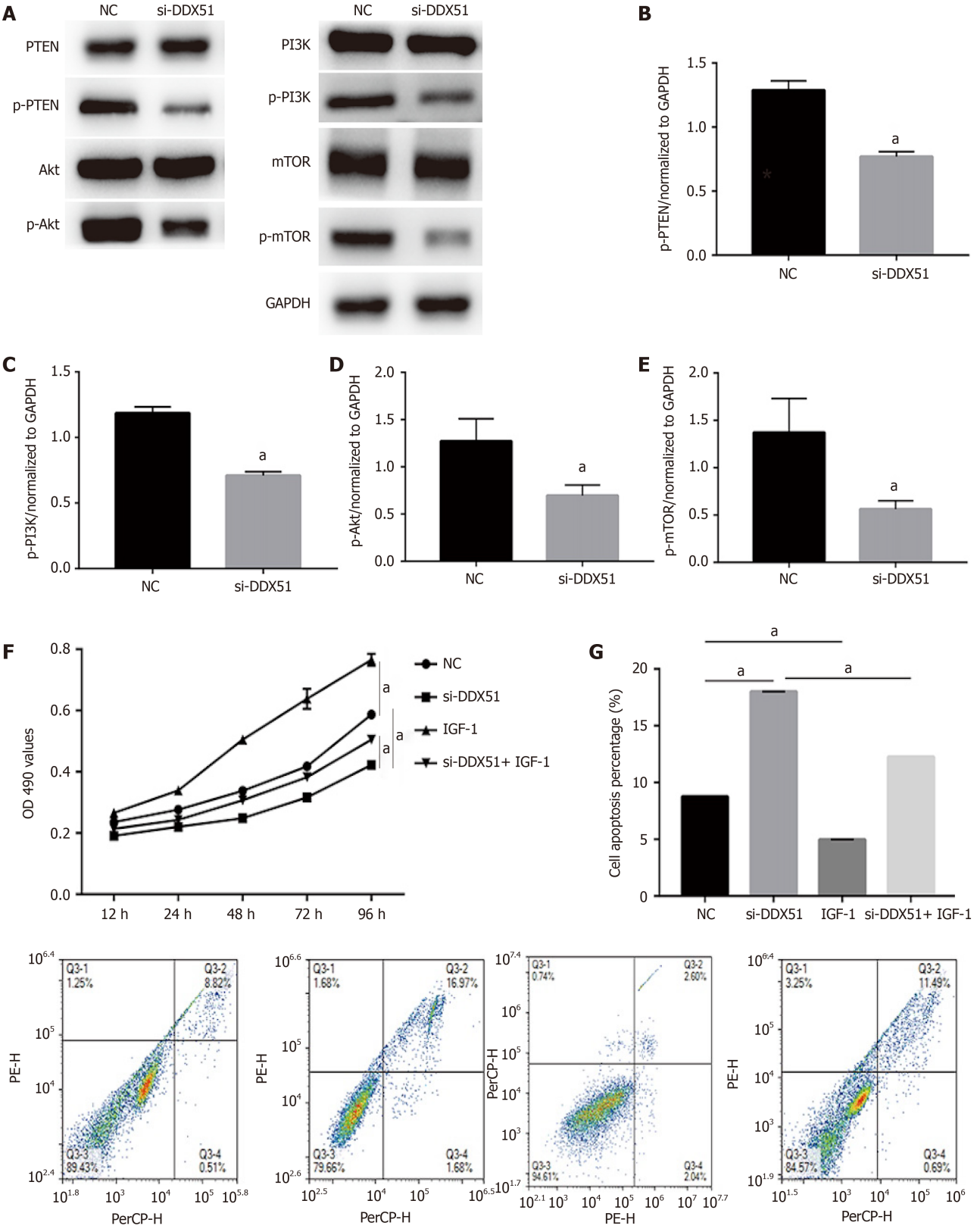

Figure 4 Knockdown of DEAD-box helicase 51 induced inactivation of the phosphoinositide 3-kinase/AKT signaling pathway. A-E: Expression of phosphoinositide 3-kinase (PI3K)/AKT pathway members, including phosphatase and tenin homolog (PTEN), phosphorylated PTEN (p-PTEN), PI3K, p-PI3K, AKT, p-AKT, mammalian target of rapamycin (mTOR) and p-mTOR, was detected by western blot analysis; F: AKT activator insulin-like growth factor 1 (IGF-1) reversed the inhibitory effect of DDX51 knockdown on the proliferation of TE-1 cells; G: AKT activator IGF-1 reversed the pro-apoptotic effect of DDX51 knockdown on the proliferation of TE-1 cells. $^{a}P < 0.05$. All data were obtained from at least three independent experiments. Negative control was a scrambled small interfering RNA.

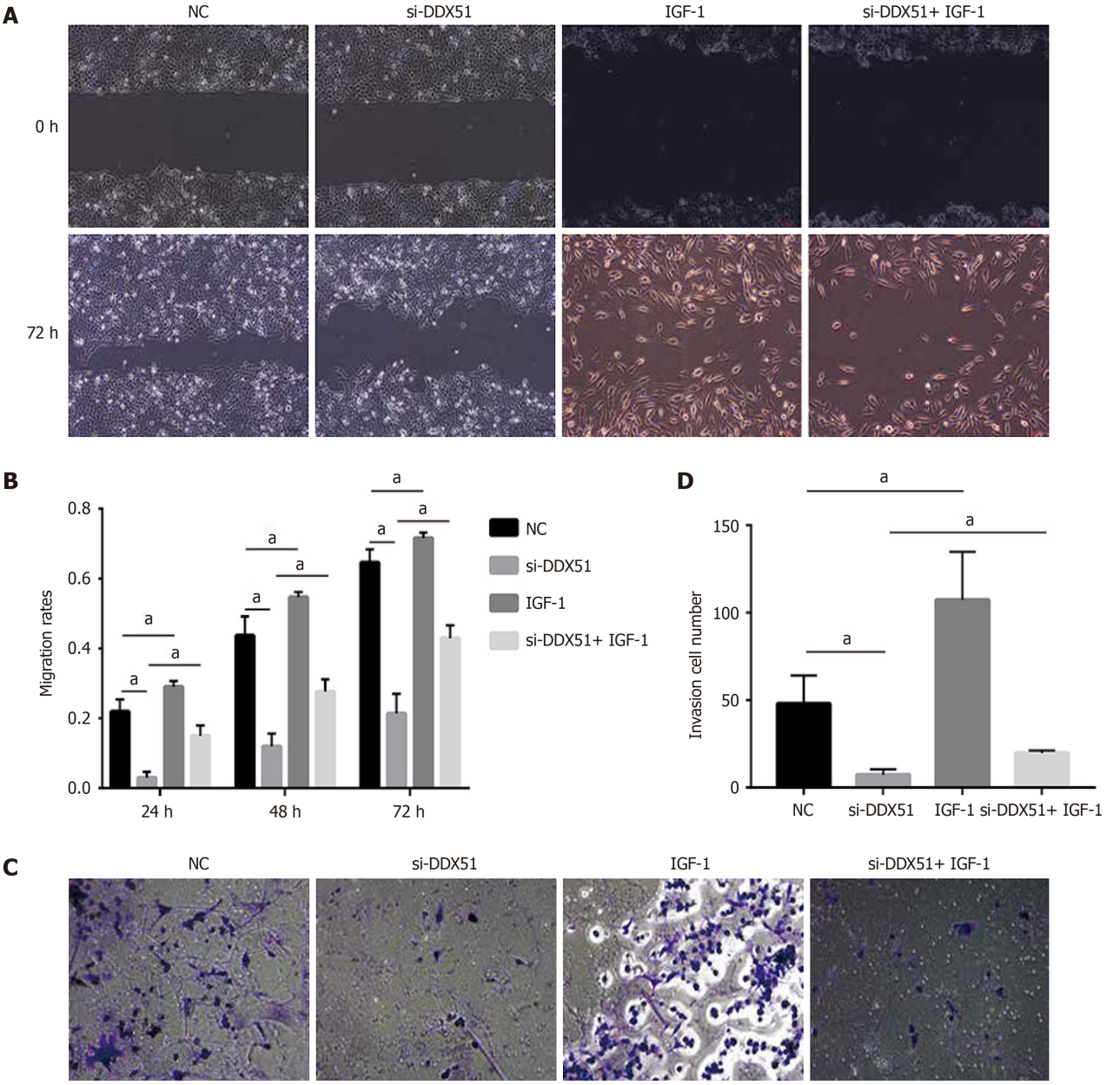

Figure 5 Anti-migration and anti-invasion effects of DEAD-box helicase 51 knockdown was mediated by the AKT pathway. A and B: AKT activator insulin-like growth factor 1 (IGF-1) reversed the anti-migration effect of DDX51 knockdown on the proliferation of TE-1 cells; C and D: AKT activator IGF-1 reversed the anti-invasion effect of DDX51 knockdown on the proliferation of TE-1 cells. $^{a}P < 0.05$. All data were obtained from at least three independent experiments. Negative control was a scrambled small interfering RNA.

further confirm the effect of DDX51 knockdown on lung and lymph node metastases. Second, the exact regulatory mechanism of DDX51 in ESCC was not explored. Future studies are required to further confirm the potential of DDX51 as a therapeutic target for the targeted treatment of ESCC.

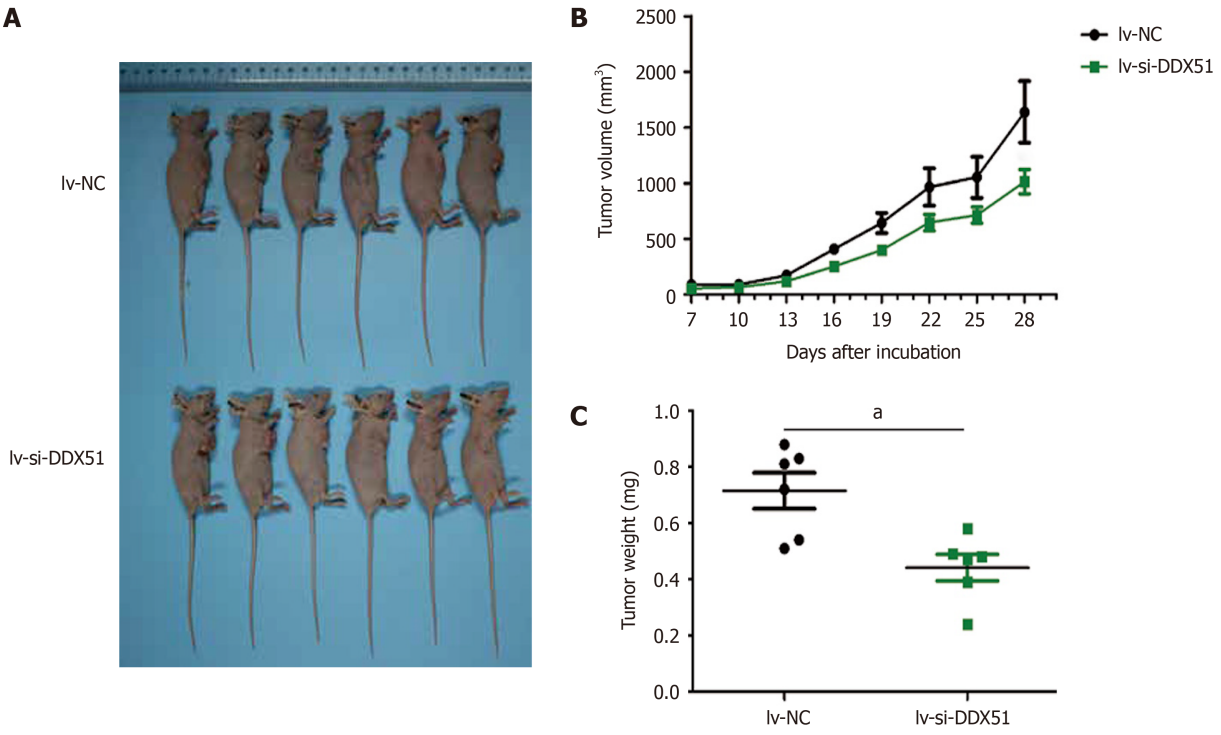

Figure 6 DEAD-Box helicase 51 knockdown inhibited the tumor growth of esophageal squamous cell carcinoma *in vivo*. TE-1 cells transfected with lentivirus-small interfering-negative control (lv-si-NC) (n = 6) or lv-si-DEAD-box helicase 51 (DDX51) (n = 6) were injected into nude mice and tumor volumes were monitored. A: The photographs of mice with esophageal squamous cell carcinoma tumor of TE-1 cells transfected with lv-si-NC or lv-si-DDX51; B: Tumor growth curves; C: At the end of experiments, mice were executed, tumors were excised and tumor weights were measured. $^{a}P < 0.05$. Negative control was a scrambled siRNA.

ARTICLE HIGHLIGHTS

Research background

Esophageal squamous cell carcinoma (ESCC) is one of the most prevalent malignancies that seriously threatens people's health worldwide. DDX51 is a member of the DEAD-box (DDX) RNA helicase family, which drives or inhibits tumor progression in multiple cancer types.

Research motivation

To identify the role of DDX51 in ESCC and the molecular mechanisms involved.

Research objectives

To explore the effect of DDX51 on ESCC progression.

Research methods

The expression of DDX51 in ESCC tumor tissues and adjacent normal tissues was detected by immunohistochemistry analysis and quantitative PCR (qPCR). We knocked down DDX51 in ESCC cell lines using small interfering RNA (siRNA) transfection. The proliferation, apoptosis, and mobility of DDX51 siRNA-transfected cells were detected. The effects of DDX51 on the phosphoinositide 3-kinase (PI3K)/AKT pathway were investigated using western blot analysis. A mouse xenograft model was established to investigate the effects of DDX51 knockdown on ESCC tumor growth.

Research results

DDX51 exhibited high expression in ESCC tissues compared with normal tissues and was associated with a poor prognosis in patients with ESCC. Knockdown of DDX51 induced inhibition of ESCC cell proliferation and promoted apoptosis. Moreover, DDX51 siRNA-expressing cells also exhibited lower migration and invasion rates. Investigation into the mechanism of action suggested that DDX51 knockdown induced inactivation of the PI3K/AKT pathway including decreased phosphorylation levels of PTEN, PI3K, AKT and mTOR. Rescue experiments demonstrated that the AKT

activator insulin-like growth factor 1 could reverse the inhibitory effects of DDX51 on ESCC malignant development. Finally, we injected DDX51 siRNA transfected TE-1 cell into an animal model, which resulted in slower tumor growth.

Research conclusions

Our study suggests for the first time that DDX51 contributes to ESCC cell proliferation by regulating the PI3K/AKT signaling pathway.

Research perspectives

DDX51 may serve as a potential therapeutic target for the treatment of ESCC.

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ORIGINAL ARTICLE

Basic Study

Fibrinogen-like protein 2 deficiency inhibits virus-induced fulminant hepatitis through abrogating inflammatory macrophage activation

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Author contributions: Wan XY and Xiao F wrote the manuscript; Wan XY, Ning Q and Wang HW designed projects, interpreted data and developed concept of the project; Xiao F, Hu JJ and Weng XX did the experiments; Tao R and Wang P collected samples; Xi D, Wan XY, Wang XJ and Luo XP participated the conceptual discussion of the paper; Ning Q and Wang HW revised the manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board at Tongji Hospital, Wuhan Province.

Institutional animal care and use committee statement: All animal experiments were approved by

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Abstract

BACKGROUND

Heterogeneous macrophages play an important role in multiple liver diseases, including viral fulminant hepatitis (VFH). Fibrinogen-like protein 2 (FGL2) is expressed on macrophages and regulates VFH pathogenesis; however, the underlying mechanism remains unclear.

AIM

To explore how FGL2 regulates macrophage function and subsequent liver injury during VFH.

METHODS

Murine hepatitis virus strain 3 (MHV-3) was used to induce VFH in FGL2-deficient (*Fgl2-/-*) and wild-type (WT) mice. The dynamic constitution of hepatic macrophages was examined. Adoptive transfer of *Fgl2-/-* or WT bone marrow-derived macrophages (BMDMs) into WT recipients with macrophages depleted prior to infection was carried out and the consequent degree of liver damage was compared. The signaling cascades that may be regulated by FGL2 were detected in macrophages.

RESULTS

institutional Animal Care and Use Committee of Tongji Hospital (permit number: TJ-A20171008), and conducted in accordance with state guidelines from the Ministry of Science and Technology of China.

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

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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

Following MHV-3 infection, hepatic macrophages were largely replenished by proinflammatory monocyte-derived macrophages (MoMFs), which expressed high levels of FGL2. In *Fgl2-/-* mice, the number of infiltrating inflammatory MoMFs was reduced compared with that in WT mice after viral infection. Macrophage depletion ameliorated liver damage in WT mice and further alleviated liver damage in *Fgl2-/-* mice. Adoptive transfer of *Fgl2-/-* BMDMs into macrophage-removed recipients significantly reduced the degree of liver damage. Inhibition of monocyte infiltration also significantly ameliorated liver damage. Functionally, *Fgl2* deletion impaired macrophage phagocytosis and the antigen presentation potential and attenuated the proinflammatory phenotype. At the molecular level, FGL2 deficiency impaired IRF3, IRF7, and p38 phosphorylation, along with NF-κB activation in BMDMs in response to viral infection.

CONCLUSION

Infiltrated MoMFs represent a major source of hepatic inflammation during VFH progression, and FGL2 expression on MoMFs maintains the proinflammatory phenotype *via* p38-dependent positive feedback, contributing to VFH pathogenesis.

Key Words: Viral fulminant hepatitis; Fibrinogen-like protein 2; Proinflammatory macrophages; Infiltrating macrophages; P38

Core Tip: In this study, we demonstrate that: (1) Monocytes infiltrating the liver represent a major source of hepatic inflammation, which has a decisive effect on the pathogenesis of viral fulminant hepatitis; (2) Viral infection induces FGL2 expression on macrophages, which is required for maintaining the inflammatory phenotype and cell function; and (3) FGL2 generates a positive feedback loop of an inflammatory cascade in macrophages in response to viral infection.

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INTRODUCTION

Viral fulminant hepatitis (VFH) is a type of acute liver failure (ALF) that can develop after viral infection in patients with hepatitis A, hepatitis B, and hepatitis E. VFH is a devastating syndrome characterized by severe liver injury with coagulation abnormalities and hepatic encephalopathy[1]. Pathologically, acute liver injury and subsequent massive hepatocyte loss are caused by hyperactivation of the innate immune response, followed by an excessive adaptive immune response to viral infection, leading to systemic inflammation. Innate immune cells, activated by pathogen-associated molecular patterns (PAMPs) from invading pathogens, damage-associated molecular patterns (DAMPs) released from necrotic cells, and afferent endotoxins from the portal vein, are dominant in conditions of sterile inflammation and non-virus-associated ALF[2,3]. Adaptive immunity activated by PAMPs seems to play a more important role in VFH than innate immunity because of the exposure to abundant viral antigens presented by antigen-presenting cells[4,5]. However, the extent of the contribution of innate immunity in generating viral antigens or DAMPs in VFH is not well defined.

Liver-resident macrophages, also known as Kupffer cells (KCs), are the largest population of hepatic immune cells that play an essential role in maintaining liver homeostasis and ensure rapid responses to hepatic insults[6]. Monocyte-derived macrophages (MoMFs) are an ontologically distinct subpopulation of hepatic

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macrophages, which are found in only small numbers under physiological conditions and then serve to replenish the macrophage population during liver injury[7]. Macrophages are generally classified into proinflammatory (M1) and anti-inflammatory (M2) macrophages (or alternatively activated macrophages), phenotypically corresponding to T helper (Th)1 and Th2 responses, respectively. However, the spectrum of macrophage classification remains obscure as the transition between macrophage subtypes occurs in response to microenvironment mediators[8,9]. Dynamic alterations in KC and MoMF populations were observed in the context of sterile inflammation in an experimental model of ALF induced by acetaminophen overdose, with replacement of KCs by MoMFs during disease progression[10]. Further analyses of this model revealed differential subsets of macrophages showing the protective and detrimental characteristics of KCs and MoMFs during the course of acetaminophen-induced ALF[10,11]. Moreover, an increase in the number of MoMFs with potent activity has been observed in non-pregnant patients with hepatitis E-associated ALF compared with that in pregnant patients. In a model of experimental VFH, hepatic leukocyte numbers were found to increase at an early stage of liver injury[12], suggesting the involvement of macrophages in disease progression. However, further investigation is needed to elucidate the precise roles of the distinct subpopulations of hepatic macrophages in VFH.

Fibrinogen-like protein 2 (FGL2), a membrane protein with prothrombinase at the N-terminal, is expressed in a variety of cells, such as macrophages, dendritic cells, and endothelial cells, and can be induced robustly and exclusively in macrophages in response to stimulation with cytokines [interferon (IFN)-gamma or tumor necrosis factor (TNF)-α), viral infection, and lipopolysaccharide (LPS)[7,13]. This suggests that FGL2 itself is a critical mediator of inflammation in that the interaction between inflammation and coagulation is reciprocally exacerbated in terms of inflammatory cytokine production and tissue factor secretion[11]. FGL2 deficiency was shown to prevent the development of VFH in mice following experimental murine hepatitis virus strain 3 (MHV-3) infection; this effect was speculated to be mainly dependent on its ability to regulate procoagulant activity, as evidenced by concomitant expression of FGL2 and fibrinogen production[14]. Emerging evidence suggests that FGL2 is a downstream effector molecule in the inflammatory cascades associated with VFH development and progression, which involve C5aR, TNF-α, and MSR1[7,12,15,16]. Interestingly, complement activation, a critical step for inflammation initiation, has also been observed in FGL2-deficient mice following MHV-3 infection[16], highlighting a complex regulatory network of inflammation involving FGL2 and other effectors. Therefore, we hypothesized that FGL2 plays a role in the reconstitution of hepatic macrophages to regulate proinflammatory macrophage activation that contributes to VFH progression and exacerbation. To test this hypothesis, we first explored the hepatic macrophage composition and expression pattern of FGL2 during the progression of VFH in patients with and without hepatitis B virus (HBV)-associated acute-on-chronic liver failure (ACLF), since the pathology of ACLF closely resembles that of ALF[17]. To explore the role of FGL2 in VFH progression and the underlying mechanism, we determined alterations in hepatic macrophage populations during progression of VFH in FGL2-deficient (*Fgl2*-/-) and wild-type (WT) mice intraperitoneally injected with MHV-3 at different stages after infection.

MATERIALS AND METHODS

Ethics statement

All studies on human subjects were approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology (permit number: TJ-C20170924), and all participants provided informed consent in compliance with the Helsinki Declaration revised in 1983.

The animal experiments were approved by the Institutional Animal Care and Use Committee of Tongji Hospital (permit number: TJ-A20171008) and were conducted in accordance with state guidelines from the Ministry of Science and Technology of China.

Patient samples

Seven patients with ACLF, as defined by the Asian Pacific Association for Study of the Liver (APASL) guidelines[18], who were undergoing liver transplantation were included in the study; liver samples were obtained during the surgery. The clinical features of the patients are summarized in Table 1. Control samples (n = 3) were

Table 1 Clinical characteristics of patients and controls

Characteristics	ACLF (*n* = 7)	Healthy controls (*n* = 3)
Age (year)	47.14 ± 3.3[a]	30.33 ± 3.4
Sex (male:female)	6:1	3:0
Laboratory evaluation		
ALT (U/L)	642.9 ± 360.3	8.67 ± 1.76
Tbil (μmol/L)	429.8 ± 70.53[a]	17 ± 2.34
PTA (%)	30.29 ± 3.3[a]	118 ± 12.17
HBsAg (ng/mL)	3870 ± 1600	0
HBV-DNA (10^6 copies/mL)	8.18 ± 6.78	0
Ascites and/or Encephalopathy	7	0

[a]$P < 0.5$ *vs* controls.

Age, Serum alanine aminotransferase, total bilirubin, prothrombin time activity shown as mean ± SD. ACLF: Acute on chronic liver failure; ALT: Alanine aminotransferase; Tbil: Total bilirubin; PTA: Prothrombin time activity.

obtained from a healthy liver donor and from the paratumor tissues of a patient with hepatic hemangioma and a patient with hepatocellular carcinoma without cirrhosis.

Animals, viral infection, macrophage depletion, and inhibitor treatment

Female BALB/c mice (HFK Bioscience Company Ltd., Beijing, China) at 6-8 weeks of age were used in all animal experiments. The mice were housed in a controlled environment (specific pathogen-free, 12 h light/dark cycle, 21 ± 2 °C, humidity 50 ± 10%) and had free access to food and water. *Fgl2-/-* mice were generated by introducing a deletion at the N-terminal of the open reading frame of the *Fgl2* locus using CRISPR/Cas9 technology. WT BALB/c mice were used as controls. To establish the fulminant hepatitis model, 100 plaque-forming unit (PFUs) of MHV-3 (American Type Culture Collection, Manassas, VA, USA) dissolved in 200 μL saline was injected into the WT and *Fgl2-/-* mice intraperitoneally. To deplete macrophages, mice were intravenously injected with 200 μL clodronate liposomes or phosphate-buffered saline (PBS)-liposomes as a control (Liposome B.V., the Netherlands) 24 h prior to MHV-3 injection. To inhibit MoMF infiltration, a chemokine receptor-2 (CCR2) inhibitor (cenicriviroc; 10 mg/kg) was intraperitoneally injected 24 h prior to MHV-3 infection. WT or *Fgl2-/-* mice were randomly selected to be sacrificed at 0, 24, 48, and 72 h following MHV-3 injection, and liver and blood samples were harvested for analysis.

Cell isolation

Hepatic mononuclear non-parenchymal cells from WT and *Fgl2-/-* mice were isolated *via* liver perfusion, enzymatic digestion, and differential centrifugation, as described previously[19]. Peritoneal exudate macrophages (PEMs) were isolated from the mice 3 d after intraperitoneal injection of 3% starch broth (1 mL/mice). Bone marrow-derived macrophages (BMDMs) were differentiated from hematopoietic stem cells isolated from the femurs cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and macrophage-colony stimulating factor (30 μg/mL, Peprotech) for 7 days. BMDMs or PEMs were maintained in DMEM supplemented with 10% FBS and treated with LPS (100 ng/mL), interleukin (IL)-4 (20 ng/mL), or MHV-3 (5×10^5 PFU/mL).

Macrophage adoptive transfer

KCs are the largest population of hepatic resident non-parenchymal cells and the first line of defense against pathogens and toxins[6]. The priming of KCs may therefore be involved in initiation of the hepatic immune response, a critical step for subsequent exacerbation of inflammatory accumulation. Accordingly, we also assessed liver damage following MHV-3 infection with depletion of macrophages by clodronate liposomes. WT mice were treated with 200 μL clodronate liposomes or PBS-liposomes as a control to deplete macrophages, which then served as the recipient mice for adoptive transfer. Harvested BMDMs from WT and *Fgl2-/-* mice were intravenously injected into the recipient mice at 5×10^5 cells/mouse. MHV-3 (100 PFU/mouse)

injection was performed 24 h later. Liver and blood samples were harvested at 48 h post MHV-3 infection for further analyses.

Flow cytometry

Single-cell suspensions were blocked with 2 μg/mL anti-CD16/CD32, followed by staining with fluorescence-conjugated antibody cocktails. For intracellular staining, surface-stained cells were fixed and permeabilized using Cytofix/Cytoperm Fixation/Permeabilization Kit (No. 554714, BD Pharmingen) prior to incubation with target antibodies. Data were acquired on a BD FACS Canto II flow cytometer (BD Bioscience, San Jose, CA) and analyzed using FACS Diva software.

Detailed procedures and other materials are described in the supporting information.

Statistical analyses

Statistical testing was performed using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, United States). Comparisons between WT and *Fgl2*$^{-/-}$ mice (or other situations where only two groups are compared) were performed using Student's unpaired t-test; one-way analysis of variance with Tukey's *post-hoc* test or Mann-Whitney *U* test was used for multiple comparisons (unless otherwise indicated). Data are presented as mean ± standard deviation; $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

The number of proinflammatory macrophages with elevated FGL2 expression increases in patients with hepatitis virus-induced liver failure

Marked infiltration of leukocytes was observed in samples of the patients with ACLF, along with massive necrosis in the parenchymal lobules (Figure 1A). CD68+ macrophages markedly accumulated in the periportal areas (Figure 1B, Supplementary Figure 1), suggesting widespread liver damage[20]. Furthermore, macrophages were polarized to proinflammatory macrophages, as confirmed by the expression of S100A9 (Figure 1C), an alarmin molecule expressed in proinflammatory macrophages, activated neutrophils, and granulocytes, which is often used as a biomarker of inflammation[21]. Interestingly, the majority of S100A-positive activated proinflammatory macrophages also expressed FGL2 in consecutive sections of liver samples from patients with HBV-related liver failure (Figure 1D). These data suggest that a high expression level of FGL2 could be an important molecular event of hepatic macrophage polarization during viral liver failure.

Ly6C^{high} MoMFs dominate the hepatic macrophage population during VFH progression

In the experimental fulminant hepatitis mouse model, pathogenesis was divided into an early stage without global liver damage at 24 h post-infection of MHV-3, a progression phase with continuous enlargement of block necrosis and increased transaminase levels, and an end stage defined at 72 h post-infection when the animals were near death (Figure 2A and B). At the early stage of infection, hematoxylin and eosin staining showed that the hepatic architecture was intact and no obvious macrophages infiltration was noted (Figure 2A). Aggregating plots of the CD11b F4/80-expressing flow cytometry panel[22] showed a continuous increase in the number of hepatic MoMFs (CD11b^{high}F4/80^{int}) and a decline in the number of KCs (CD11b^{low} F4/80^{high}) over the course of infection, accompanied by enlarged lesions until confluent necrosis of the liver lobules occurred at the later stage (Figure 2A and C). Notably, KCs constituted the dominant hepatic macrophage population at the early stage of infection, with an increased M1 macrophage subset [induced nitric oxide synthase (iNOS+) KCs] (Figure 2D, Supplementary Figure 2A and C). However, MoMFs subsequently dominated the hepatic macrophage population, characterized by an increased ratio of Ly6C^{high} cells; however, the Ly6C^{high}/Ly6C^{low} ratio decreased at the end stage of the disease (Figure 2E, Supplementary Figure 2A), implying a transition from Ly6C^{high} MoMFs to Ly6C^{low} MoMFs. The dynamic alterations in hepatic macrophage populations were also revealed by an increase in the levels of proinflammatory cytokines TNF-α and IL-6 during progression, which then decreased at the end stage, along with a continued decrease in transforming growth factor (TGF)-β levels in macrophages (Supplementary Figure 2D). Consistent with the reconstitution of hepatic

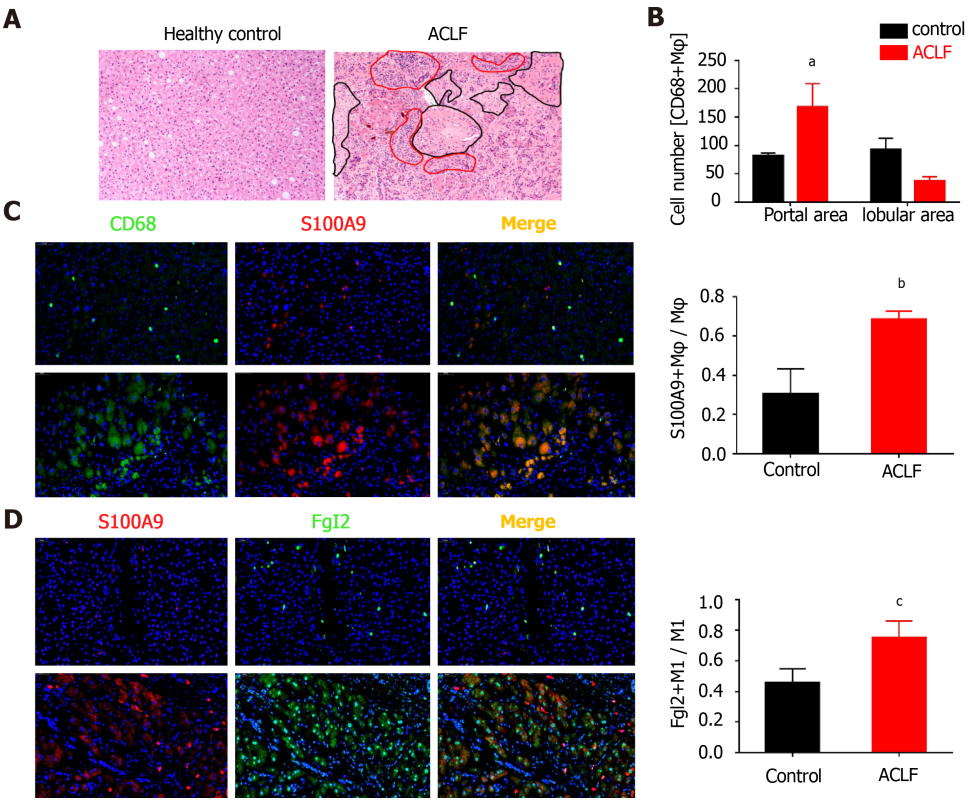

Figure 1 Fibrinogen-like protein 2 was expressed on proinflammatory macrophages in patients with hepatitis B virus-associated liver failure. A: Representative image of liver sections (200×) subjected to Hematoxylin and eosin staining (H&E); Black circles point to transparent necrosis, red circles point to infiltrating leukocytes; B: Calculation of CD68+ macrophages at periportal and lobular areas; C: Immunofluorescence against CD68, S100A9 on liver sections (200×) from patients of hepatitis B virus-associated acute chronic-on liver failure (acute-on-chronic liver failure (ACLF), *n* = 7) and healthy controls (*n* = 3); D: Immunofluorescence against S100A9 and fibrinogen-like protein 2 on liver sections (200×) from ACLF patients (*n* = 7) and healthy controls (*n* = 3), representative images on the left and statistical columns on the right. Data are expressed as mean ± SD.

macrophage populations in which Ly6C^{high} MoMFs are more inflammatory with an expanded spectrum of cytokine and chemokine expression compared with M1 macrophages[23], the serum MCP-1 level was remarkably increased during infection-induced liver failure progression (Supplementary Figure 2B).

Potent induction of FGL2 is associated with the proinflammatory phenotype of both KCs and MoMFs in response to viral infection

As shown in Figure 3A, over 90% of FGL2-positive KCs were found in iNOS+ macrophages based on flow cytometry. Moreover, Ly6C^{high} MoMFs constituted the major population of FGL2-positive MoMFs, and the expression level of FGL2 was remarkably higher in Ly6C^{high} MoMFs than in Ly6C^{low} MoMFs (Figure 3B). After viral infection, sequential induction of FGL2 expression was observed in both polarized M1 KCs and Ly6C^{high} MoMFs during disease progression, although a slight decrease in the expression level was observed when the KC and MoMF populations were somewhat exhausted at 48 and 72 h post viral infection, respectively (Figure 3C and D). However, FGL2 expression was maintained at low levels in Ly6C^{low} populations (Figure 3D). Together, these data suggested that FGL2 is markedly induced in proinflammatory macrophages upon viral infection.

FGL2 deficiency prevents proinflammatory macrophage activation after MHV-3 infection

In *Fgl2-/-* mice (Supplementary Figure 3A), liver damage following MHV-3 infection was significantly attenuated, as revealed by narrowed necrosis foci and lower alanine transaminase (ALT) and aspartate transaminase (AST) levels compared with those in WT mice (Figure 4A and B). In addition, viral titers were reduced in *Flg2*$^{-/-}$ mice compared with those in their WT counterparts (Supplementary Figure 3B). Consistent with the pathology results, monocyte infiltration was significantly reduced in *Flg2*$^{-/-}$ mice, whereas a large number of KCs was maintained during VFH progression (Figure 4C). To determine whether *Fgl2* was required for proinflammatory

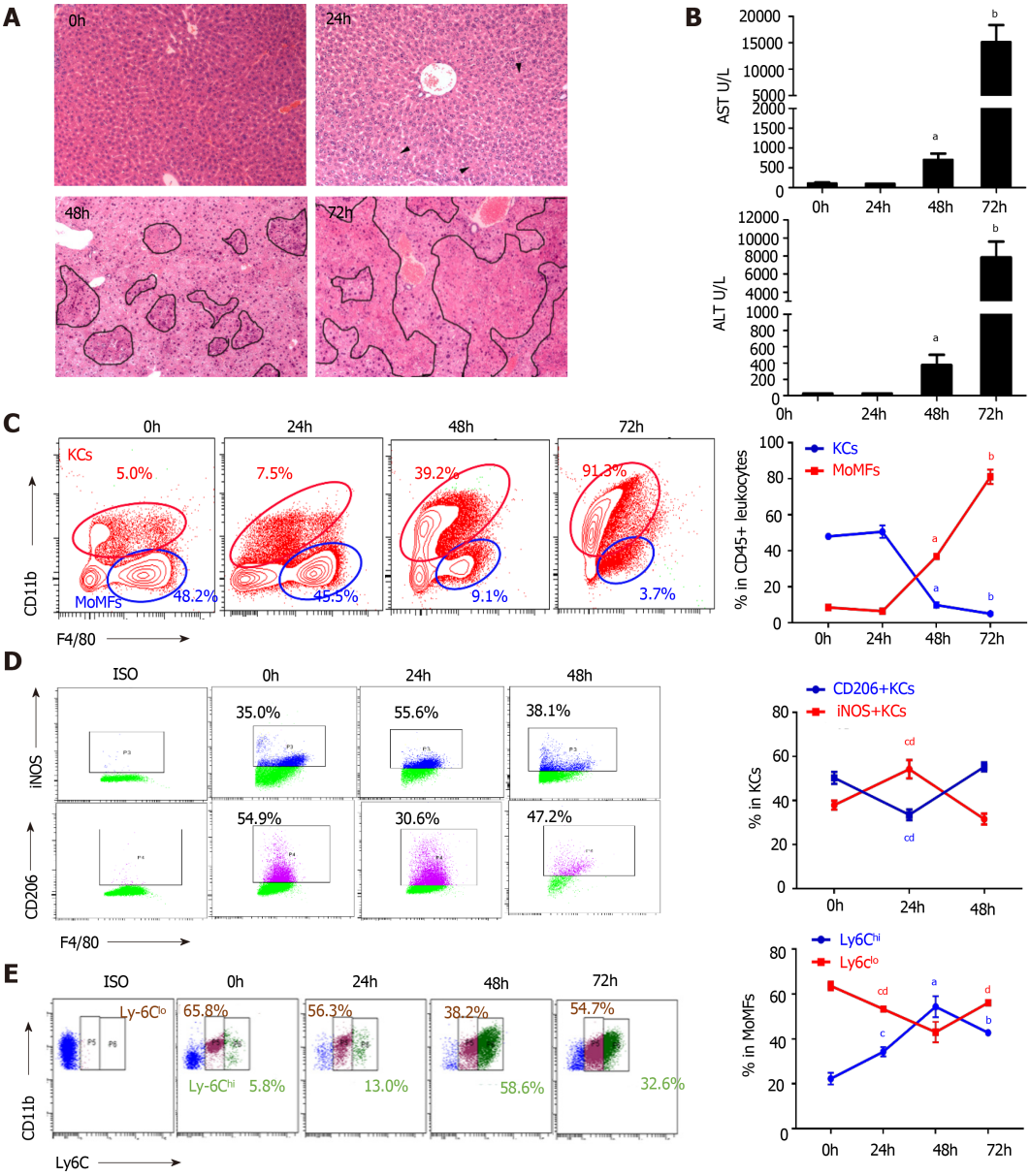

Figure 2 Dynamic alteration of macrophage subsets during viral fulminant hepatitis progression. A: Time-course H&E staining on liver sections of mice after murine hepatitis virus strain 3 infection. Arrowheads indicated hepatocyte cytoplasmic destruction, Circles pointed to necrosis; B: ALT and AST levels from serum of mice post-viral infection; C: Flow cytometry of KCs (CD45+ F4/80high), MoMFs (CD45+ Ly6C+ F4/80int) of cells (left), and their percentage in the liver at various time point after viral infection; D: Flow cytometry of KCs (left) and frequency of M1 (iNOS+), M2 (CD206+) macrophages in KCs (right); E: Representative image of MoMFs (left) and frequency of Ly6Chigh and Ly6Clow MoMFs at different time point after viral infection. Data are presented as mean ± SD (n = 5). These experiments were repeated at least three times. KCs: Kuffer cells; MoMFs: Monocyte derived macrophages.

macrophage activation, we investigated the polarization of KCs at the early stage of VFH when KC populations were reserved. We found a decrease in the M1 KC subset and an increase in the M2 KC numbers in FGL2-deficient mice before and after MHV-3 infection (Figure 4D), suggesting that FGL2 depletion impaired inflammatory macrophage activation and favored the M2 phenotype. As expected, a small Ly6C^{high} subset was also observed in the MoMF population in FGL2-deficient mice after viral infection, implying that *Fgl2* loss predisposed MoMFs to the Ly6C^{low} phenotype (Figure 4E). This interpretation was further supported by the reduced levels of TNF-α and IL-6 and the increased levels of TGF-β on KCs from *Fgl2*-/- mice after viral infection compared with those in WT mice (Supplementary Figure 3C).

FGL2 induces KC polarization for initiation of inflammatory events after viral infection

Macrophage depletion by clodronate liposomes significantly relieved liver injury, as shown by decreased necrosis foci and ALT/AST levels, in both WT and FGL2-deficient

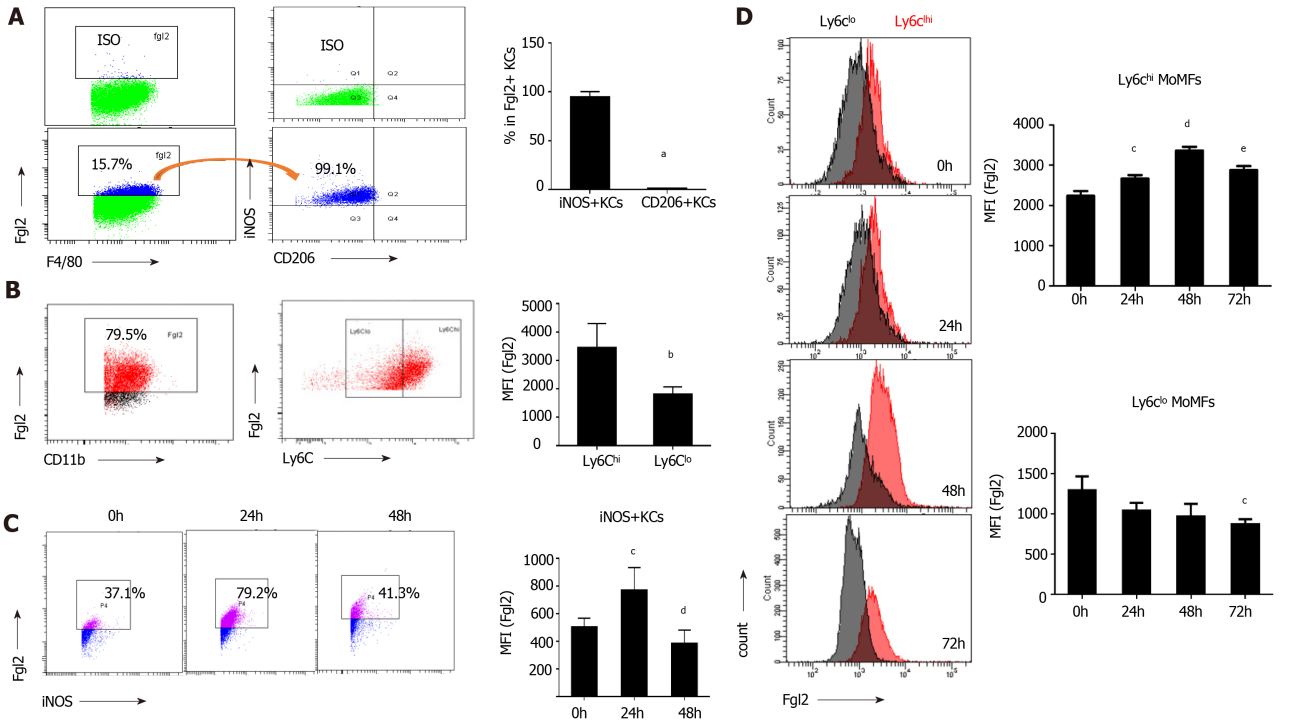

Figure 3 Fibrinogen-like protein 2 expression was robustly inducted upon proinflammatory macrophage activation. A: Flow cytometry of fibrinogen-like protein 2 (Fgl2) +KCs expressing iNOS and CD206 under homeostatic conditions (left), and frequency of iNOS+ and CD206+ cells subsets (right); B: Representative image of Fgl2+MoMFs expressing Ly6C (left), and relative expression level of Fgl2 in Ly6ChiMoMFs and Ly6Clo MoMFs under physiological conditions (right); C: Time course-presentation of Fgl2 expression in M1 polarized KCs (left), and expression level of Fgl2 in Ly6ChiMoMFs and Ly6Clow MoMFs, respectively (right); D: Cell counts of Fgl2+ MoMFs (left), and relative level of Fgl2 expression on both MoMFs subset (right) following viral infection. Data are presented as mean ± SD (n = 5). These experiments were repeated at least three times. MFI: Mean fluorescence intensity.

mice (Figure 5A and B). Notably, further reduction in liver ALT/AST levels and fewer necrotic hepatocytes were observed in FGL2-deficient mice after viral infection, suggesting the requirement of FGL2 for macrophage polarization and disease progression. To determine whether FGL2 regulates macrophage polarization directly, BMDMs from WT and *Fgl2*-/- mice were transferred into WT recipient mice with self-BMDMs depleted in advance in the VFH model. Interestingly, *Fgl2*-/- BMDMs exhibited reduced numbers of inflammatory polarized macrophages and increased numbers of anti-inflammatory macrophages (Figure 5D and E). Moreover, recipient mice adoptively transferred with *Fgl2*-/- BMDMs exhibited reduced liver injury, as revealed by histological analysis (Figure 5A) and ALT/AST levels (Figure 5C), when compared with that in recipients transferred with WT BMDMs.

As depicted in Supplementary Figure 3D and E, smaller numbers of MPO^{high} myeloid cells, which are considered to be neutrophils, were observed in liver sections from mice with macrophage depletion or in FGL2-deficient mice compared with those from untreated WT mice following MHV-3 infection. However, no significant difference in neutrophil abundance was observed between WT and *Fgl2*-/- mice with macrophage depletion.

Because MoMFs formed the largest component of myeloid cells during the acute stage and CCR2 is mainly expressed in proinflammatory monocytes (Ly6C^{high})[24], we speculated that MoMF-derived inflammation is a major source of hepatic inflammation. Indeed, treatment with the CCR2 inhibitor cenicriviroc significantly reduced the virus-induced liver damage (Figure 5F and G). Collectively, these data suggest that FGL2 directly regulates proinflammatory macrophage polarization and that infiltrated MoMFs are the major source of hepatic inflammation during acute liver injury.

FGL2 deficiency attenuates macrophage M1 polarization in vitro

To further determine whether FGL2 regulates macrophage polarization directly *in vitro*, we examined its regulatory effect on BMDMs and PEMs by LPS stimulation or MHV-3 infection. As expected, in WT BMDMs, robust production of cytokines and chemokines, such as IL-1β, TNF-α, IL-6, IL-12, and MCP-1, and reactive species, such as the NO-derived nitrite product (NO_2^-), were observed in response to either LPS treatment or MHV-3 infection. In contrast, the levels of such products were markedly

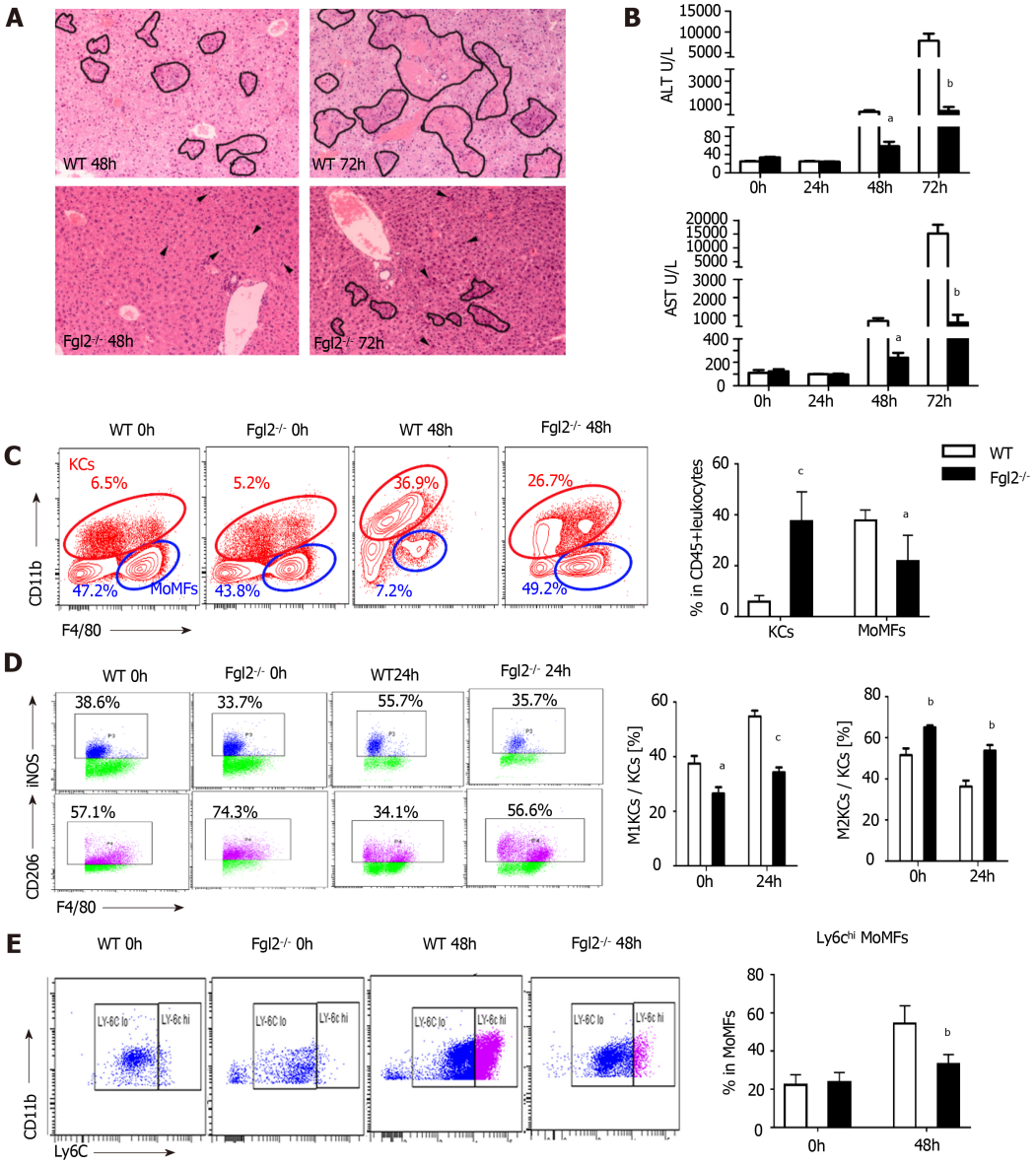

Figure 4 Fibrinogen-like protein 2 promotes pro-inflammatory macrophage polarization following murine hepatitis virus strain 3 infection.
A: H&E staining on liver sections from wild type (WT) and fibrinogen-like protein 2 (*Fgl2-/-*) mice at 48 and 72 h post viral infection. Circled field represented bulk necrosis. Arrows point to necrotic cells; B: Serum alanine transaminase and aspartate transaminase levels at 0, 24, 48, and 72 h post viral infection in WT and *Fgl2-/-* mice; C: Flow cytometry of KCs and MoMFs in hepatic CD45+ leukocytes at steady condition and 48 h following viral fulminant hepatitis in WT and *Fgl2-/-* mice (left); and respective frequency at 48 h post infection; D: Frequency of polarized M1 (iNOS+), M2 (CD206+) KCs at 0 and 24 h post murine hepatitis virus strain 3 (MHV-3) infection; E: Frequency of Ly6Chi and Ly6Clow MoMFs at 0 and 48 h post MHV-3 infection. Data are presented as mean ± SD (n = 5). These experiments were repeated at least three times.

reduced in BMDMs from *Fgl2-/-* mice under the same condition (Figure 6A). Consistently, the transcription levels of M1 indicators, such as NOS2, TNF-α, IL-6, IL-1 β, IL-12, and Marco, were significantly reduced in BMDMs of *Fgl2-/-* mice compared with those in BMDMs from WT mice in response to either LPS stimulation or MHV-3 infection (Figure 6B and C). In contrast, both IL-10 production and the transcriptional levels of M2 markers were significantly higher in *Fgl2-/-* BMDMs than in WT BMDMs in response to IL-4 treatment (Figure 6D and E). Similar results were also obtained in *Fgl2-/-* PEMs in response to LPS treatment (Supplementary Figure 4). Taken together, these data suggest that FGL2 deficiency impairs the proinflammatory polarization of macrophages and promotes an alternative activated phenotype in response to IL-4 stimulation.

FGL2 deficiency reduces the antigen presentation and phagocytosis of macrophages

A critical function of hepatic macrophages during homeostasis maintenance is to engulf pathogens, endotoxins, and debris of dead cells *via* a process called

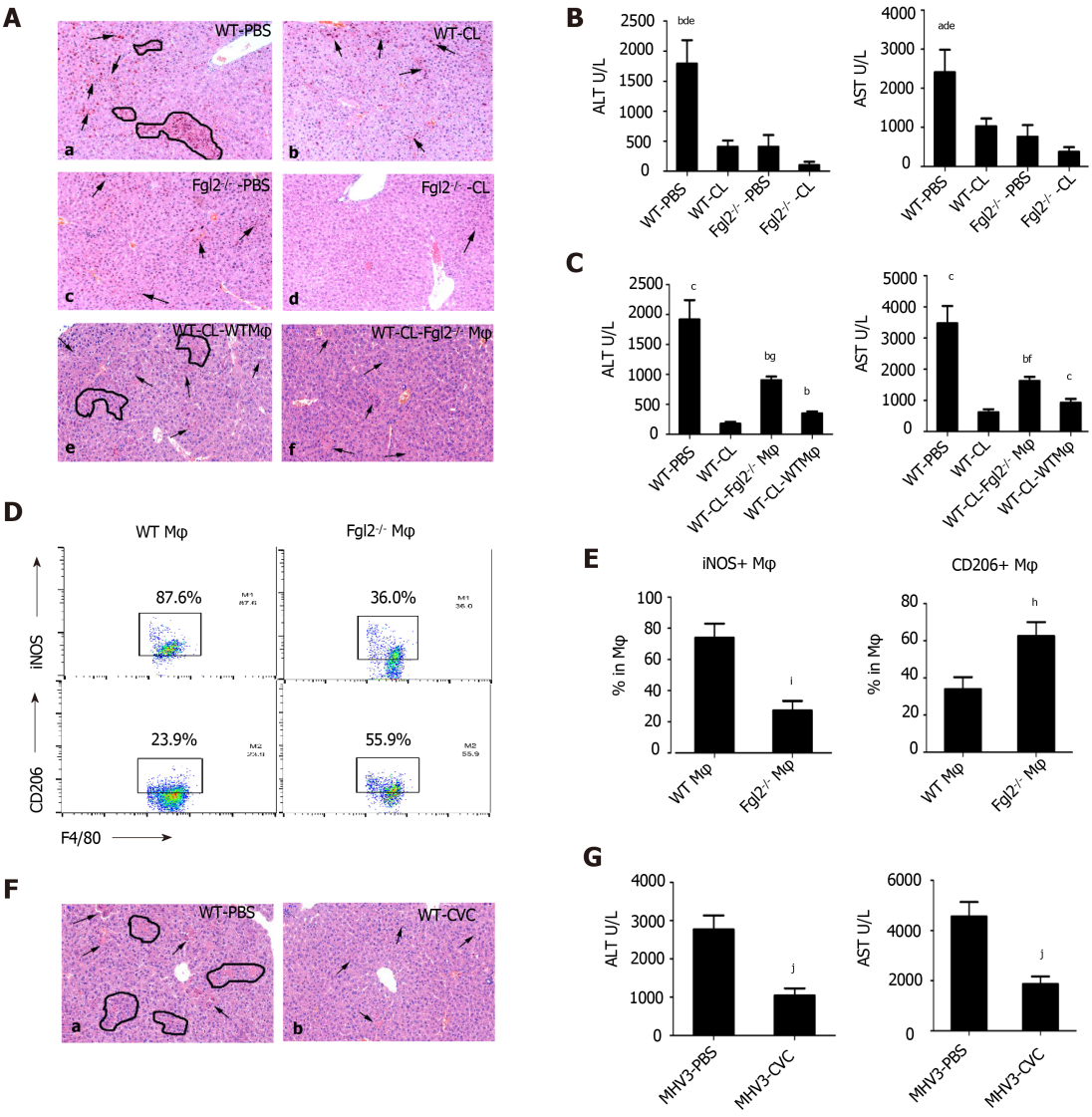

Figure 5 Depletion of macrophages in fibrinogen-like protein 2 synergically attenuated liver damage after viral infection. A: H&E staining on liver sections from mice administered with either clodronate liposomes (CL) (b and d on the right) or PBS-liposomes (PBS) (a and c on the left) at 48 h post murine hepatitis virus strain 3 (MHV-3) infection; e-f, liver sections from bone marrow-derived macrophages (BMDMs) adoptive transferred mice at 48 h post MHV-3 infection (e: WT BMDM donor; f: Fibrinogen-like protein 2 (*Fgl2*-/-) BMDM donor); B: Serum ALT and AST levels from clodronate liposomes-treated WT and *Fgl2*-/- mice at 48 h post MHV-3 infection; C: Serum AST and ALT levels from WT and *Fgl2*-/- BMDM chimeric mice; D: representative F4/80+ iNOS+ and F4/80+ CD206+ donor macrophages from WT and *Fgl2*-/- BMDM chimeric mice at 48 h post infection; E: statistical analysis of iNOS+ and CD206+ donor macrophages in chimeric mice at 48 h post infection; F: H&E-stained liver section from mice which were treated with Cenicriviroc (CVC) in advance and subjected to MHV-3 infection for 48 h. Arrows represented necrotic cells, circles represent areas of hepatocyte necrosis. Image magnificence: 200×; G: aminotransferase levels of CVC treated viral fulminant hepatitis mice and its control. Data were presented as mean ± SD (n = 5). These experiments were repeated at least three times. BMDM: Bone marrow derived macrophages.

phagocytosis, followed by antigen presentation by major histocompatibility complex (MHC) II for T cells. We therefore cultured *E. coli* labeled with red fluorescence together with peritoneal macrophages to examine their phagocytic capabilities. The number of engulfed bacteria was notably smaller in macrophages from FGL2-deficient mice than in macrophages from WT mice, although more macrophages were detected in *Fgl2*-/- mice (Figure 7A and B). Furthermore, surface expression of MHC II was more severely impaired in FGL2-deficient macrophages than in WT macrophages under normal conditions or after viral stimulation (Figure 7C), whereas expression of CD80, CD86, or MHC I was not altered (data not shown). These data suggest that FGL2 deficiency attenuates both the phagocytic capacity and antigen presentation potential of macrophages.

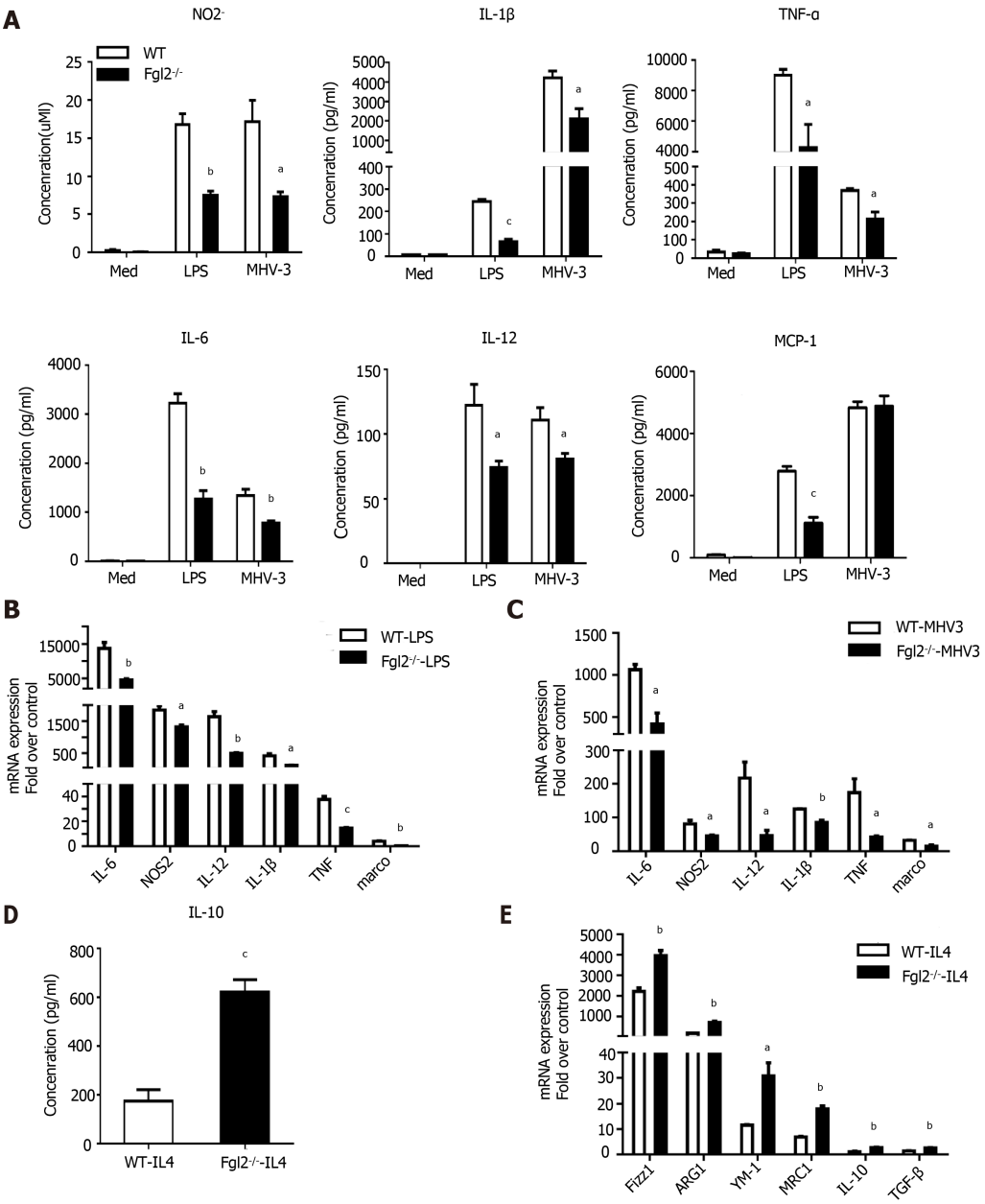

Figure 6 Fibrinogen-like protein 2 promoted bone marrow-derived macrophages M1 polarization *in vitro*. A: Supernatant concentration of NO2-, IL-1β, TNF-α, IL -6, IL-12 and MCP-1 released by bone marrow-derived macrophages (BMDMs) after LPS (100 ng/mL) treatment or murine hepatitis virus strain 3 (MHV-3) infection for 24 h; B: Relative transcriptional level of M1 markers (NOS2, IL-6, IL-12, IL-1β, TNF-α and marco) of WT and fibrinogen-like protein 2 (*Fgl2-/-*) BMDMs after the stimulation of LPS for 6 hours; C: Relative transcriptional level of M1 markers of WT and *Fgl2-/-* BMDMs after MHV-3 infection for 8 hours; D: Supernatant IL-10 concentration of BMDMs culture after the IL-4 stimulation (20 ng/mL) for 72 h; E: mRNA expression of M2 markers (Fizz1, ARG1, YM-1, MRC1, IL-10 and TGF-β) of WT and Fgl2 -/- BMDMs after of IL-4 treatment for 72 h. All data are presented as mean ± SD (n > 3). These experiments were repeated at least three times.

FGL2 modulates the inflammatory signaling cascade by activating NF-κB, IRF3, IRF7, and p38

To explore how FGL2 modulates inflammatory cascades, we examined the serial components of inflammatory signaling pathways in BMDMs by mimicking invading pathogen infections *in vitro*. Consistent with the results from MoMFs in the VFH model, FGL2 was robustly induced in BMDMs following LPS treatment and MHV-3 infection (Figure 8A). In addition, phosphorylation of IκBα, p65, IRF3, and IRF7, which are critical modulators of NF-κB activation and IFN expression, was remarkably impaired in *Fgl2-/-* BMDMs in response to LPS treatment and viral infection (Figure 8B, Supplementary Figure 5). However, the expression of adaptor proteins such as TRIF and MyD88 was not altered under the same conditions (Figure 8B). Among mitogen-activated protein kinases (MAPKs), the level of phosphorylated p38 was reduced, whereas the levels of activated (phosphorylated) JNK and ERK were not

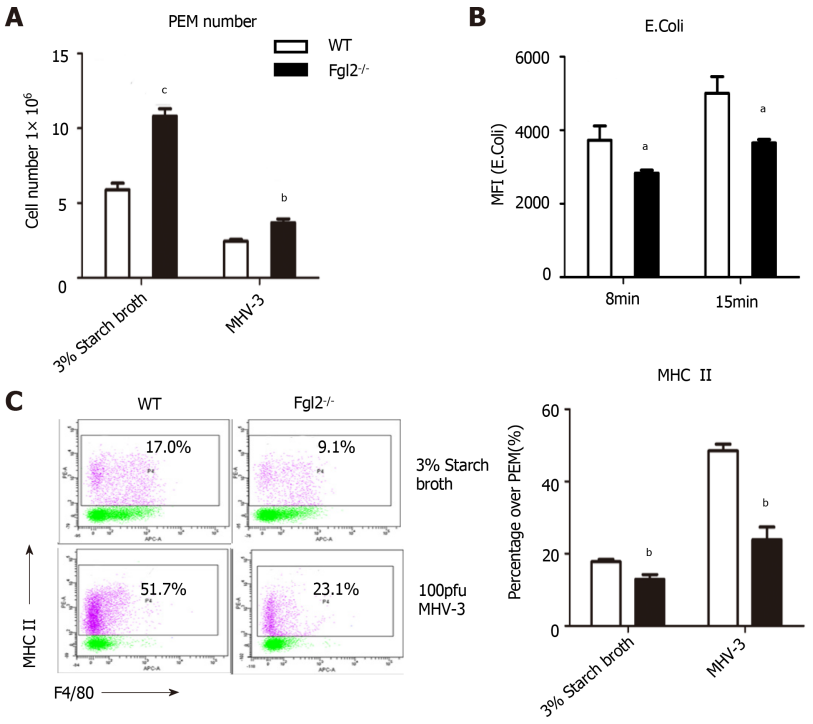

Figure 7 Fibrinogen-like protein 2 deficiency resulted in low expression of major histocompatibility complex II and impaired phagocytosis of macrophage. A: Absolute number of PEMs under the stimulation of 3% starch broth or murine hepatitis virus strain 3 (MHV-3) extracted from wild-type (WT) and fibrinogen-like protein 2 (*Fgl2-/-*) mice; B: MFI of FITC+ phagocyted *E.Coli* by PEMs from WT and *Fgl2-/-* mice at different time points; C: Expression of major histocompatibility complex II on WT and *Fgl2-/-* PEMs under the stimulation of 3% starch broth or MHV-3 infection. All data are presented as mean ± SD. These experiments were repeated at least three times. PEMs: Peritoneal exudate macrophages.

affected in *Fgl2-/-* BMDMs compared with those in WT BMDMs following LPS treatment and viral infection (Figure 8C, Supplementary Figure 5), suggesting that FGL2 induces a positive feedback proinflammatory loop through p38 activation. Phosphorylation of p65 at Ser276 by c-Raf1 enables the recruitment of acetyltransferase CREB-binding protein (CBP) and p300, which leads to the acetylation of p65 and thus an enhanced NF-κB transcription rate[16]. Interestingly, phosphorylation of c-Raf1 and p65 (Ser276) and acetylation of p65 were reduced in *Fgl2-/-* BMDMs compared with those in WT BMDMs following either LPS stimulation or viral infection (Figure 8D). Phosphorylation of TBK, a modulator downstream of SYK that is involved in C-type lectin receptor (CLR)/Toll-like receptor (TLR) signaling-mediated innate immunity [25], did not differ between WT and *Fgl2-/-* BMDMs following LPS and MHV-3 challenge (Figure 8E). Taken together, our data suggest that induction of FGL2 in macrophages regulates inflammatory signaling by modulating p38, IRF3, IRF7, and p65 phosphorylation.

DISCUSSION

Fulminant hepatitis, the most severe form of acute viral hepatitis, is a type of ALF induced by viral infection. Mounting evidence suggests that leukocyte infiltration initiates inflammation and liver damage in chronic viral hepatitis and alcoholic and non-alcoholic hepatitis with acute liver injury. However, there is no ideal experimental model to completely mimic the specific clinical manifestations of acute viral hepatitis. Most studies to date have focused on acute hepatitis B, in which virus-specific cytotoxic T lymphocytes (CTLs) mediate liver damage subsequent to viremia[26,27]. Similarly, non-virus-specific CD8+ T cells with innate-like cytolytic activity promote liver damage in patients with acute hepatitis A[28]. The innate immune response is involved in the early stage of viral clearance. Among innate immune cells, natural killer (NK) cells are preferentially studied because of their potent capacity to direct viral clearance as well as liver injury. Evidence from experimental VFH models and patients with acute viral hepatitis has revealed the detrimental contribution of NK cells to disease outcome[29,30]. Macrophages are the largest population of non-parenchymal immune cells in the liver, which proliferate and are activated in patients

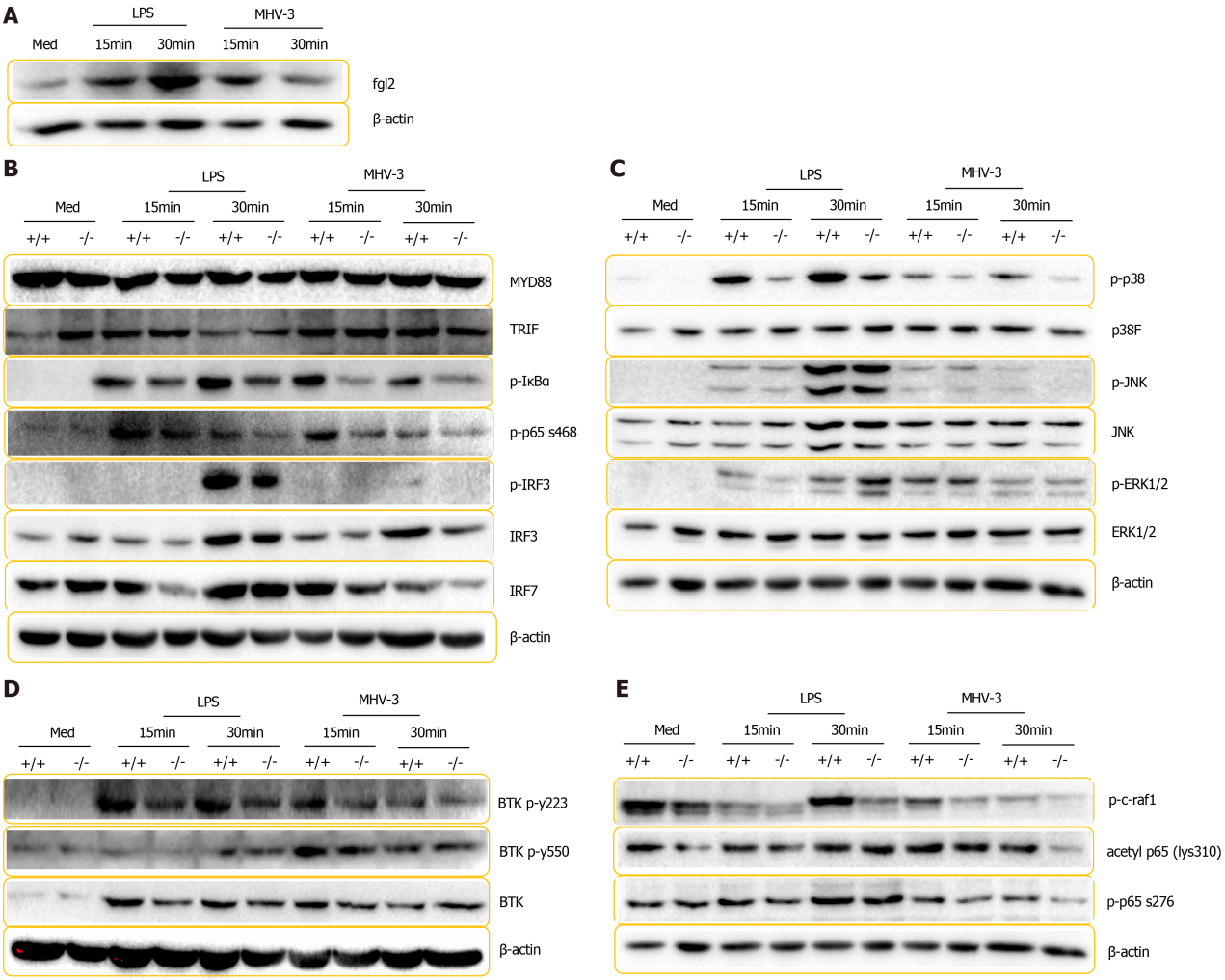

Figure 8 Fibrinogen-like protein 2 deficiency impaired inflammatory cascade in response to lipopolysaccharide treatment of viral infection. A: Immunoblot for fibrinogen-like protein 2 (Fgl2) and β-actin expression in wild-type (WT) BMDMs in response lipopolysaccharide (LPS) stimulation and murine hepatitis virus strain 3 (MHV-3) infection. B-E: Immunoblot for proteins panels from lysates of WT and *Fgl2-/-* BMDMs in response to LPS treatment and MHV-3 infection; B: MyD88, TRIF, IκBα, phosphorylated IκBα, phosphorylated p65 (S468) and IRF3, phosphorylated IRF3, and IRF7; C: p38, JNK, ERK and phosphorylated p38, JNK, ERK; D: Phosphorylated c-Raf, phosphorylated p65 (S276), acetyl p65 (lys310); E: BTK, and phosphorylated BTK (Y223), BTK (Tyr550). These experiments were repeated at least three times.

with acute hepatitis E virus infection and associated ALF, although their function is somewhat impaired[31]. The development of VFH is associated with hepatic macrophage replenishment by infiltrating macrophages, suggesting that macrophage infiltration is a fundamental event in VFH progression[32]. However, the pathological involvement of innate immunity in the liver damage occurring during acute viral hepatitis is not fully understood.

Experimental VFH established by either the coronavirus MHV-3 or infection with other hepatitis-causing viruses induces acute liver injury followed by ALF in 3-5 d in different strains of mice[12,14]. This suggests that adaptive immunity may not be the central event in disease progression, because of the rapid mortality despite infiltration of T cells to the liver[33]. In addition to monocytes, dendritic cells and neutrophils are also recruited to the liver during the progression of VFH, although the characteristics of the infiltrating leukocytes and their respective contributions to inflammation are unclear[12]. Neutrophils are a small subset of leukocytes that are required for monocyte infiltration during liver injury in mouse models of non-alcoholic steatohepatitis, acetaminophen overdose, and hepatitis virus infection[9,12,24]. In this study, we found that MoMFs dominated the population of infiltrating leukocytes, with a proinflammatory Ly6C^{high} MoMF subset comprising the majority of the population, suggesting that targeting the transition from Ly6C^{high} MoMFs to Ly6C^{low} MoMFs may be a promising strategy for treating VFH. Notably, the continued decrease in KC numbers during VFH suggests that necrosis occurred not only in hepatocytes but also in non-parenchymal cells.

FGL2 deficiency has been shown to prevent fulminant hepatitis following MHV-3 infection, which otherwise causes ALF with 100% mortality[14]. Moreover, FGL2 expression is largely distributed in and robustly induced by macrophages, in spite of moderate expression in dendritic cells and endothelial cells following viral infection [7]. The mechanism by which FGL2 contributes to VFH is generally considered to involve its procoagulant activity[14,34]. However, reciprocal activation between inflammation mediators or cytokines and tissue factor-mediated fibrin deposition and thrombin generation, the major contributor to inflammation-initiated coagulation, is largely dependent on IL-6[11,35]. It is therefore difficult to determine whether co-localization between fibrin deposition and FGL2 expression solely results from the prothrombinase activity of FGL2. In addition, inflammation may be induced by tissue factors secreted by local endothelial and immune cells[36]. Proinflammatory macrophage polarization is a process resulting from the Th1 response and pattern recognition receptor (PRR)-mediated signaling events initiated by PAMPs or DAMPs [37]. Unlike soluble FGL2 expressed by regulatory T cells and Th2-like immune cells with immunosuppressive activity, membrane FGL2 can amplify the classical inflammatory cascades and may act as a co-receptor to cooperate with TLRs or other PPRs for signal transduction. Our results suggest that FGL2 deficiency may foster a default transition from proinflammatory macrophages to an alternative activated phenotype because more M2 macrophages and Ly6C^{lo} MoMFs were observed under normal conditions. The inflammatory cytokines secreted by endothelial and dendritic cells in which FGL2 is expressed may also play a role; however, compared to macrophages, these cells are in relatively low abundance[38].

The innate immune responses triggered by viral infection are largely regulated by PPR-mediated signaling, which depends on cytosolic adaptors (*e.g.*, TRIF and MyD88) and kinase-dependent factors[39]. Macrophage M1 polarization promotes the activation and translocation of NF-κB and the IRF3/IRF7 complex to initiate the transcription of Th1-associated gene targets[40]. p38 MAPKs are known to modulate PPR signaling by collaborating with NF-κB-mediated transcription[33]. CLRs are another type of PPR that modulate TLR-mediated signaling in the defense against invading pathogens[14]. In invertebrates, numerous molecules containing fibrinogen-related domains participate in immune response transcription and have been shown to act as PPRs in defense processes, such as agglutination, and to cooperate with CLRs to synergistically clear pathogens[41]. Thus, we questioned whether FGL2 regulated TLR signaling in a CTL-like manner. MyD88-dependent PPR signaling involves activation of members of the IRAK family, which in turn stimulates the E3 ligase activity of TRAF6, enabling activation of the downstream ubiquitin-dependent kinase TAK1[42]. Upon activation, TAK1 activates MAPK and its downstream kinase IKK, which in turn phosphorylates the NF-κB inhibitor IκBα, leading to ubiquitin-dependent IκBα degradation and subsequent NF-κB activation[42]. Thus, TRIF-dependent PPR signaling involves TRAF3 recruitment and activation of TBK1 and IKK□, which stimulate IRF3 phosphorylation and NF-κB activation, thereby leading to the transcriptional induction of type I IFNs and inflammatory cytokines[43]. Membrane proteins that regulate membrane PPR-mediated signaling function as co-receptors or factors involved in TLR assembly, internalization, or trafficking[44-47]. Accordingly, FGL2 may act as an assembly factor, could interact with PPRs for ligand-receptor binding, or even mediate the trafficking between the membrane and endosome, thereby facilitating inflammatory signaling transduction. Previous data showed that MHV-3-induced FGL2 expression in macrophages relies on p38 activation[48]. In this work, we found that FGL2 is needed for p38 phosphorylation, suggesting that FGL2 expression provides a positive feedback loop for proinflammatory aggregation in macrophages.

The exact role of resident macrophages and MoMFs in VFH is obscure. We speculate that resident macrophages may initiate liver inflammation during the early stage of viral infection because MoMFs exacerbate liver damage during the acute phase. Resident macrophages represent the first line of defense against viral infection. Once primed, resident macrophages produce chemotaxins and cytokines that recruit and activate the MoMFs, and our results suggest that FGL2 expression on macrophages prompts and enhances their inflammatory phenotype.

CONCLUSION

Our data revealed, for the first time, that Ly6C^{high} MoMF infiltration is a critical event for hepatic inflammatory accumulation and subsequent liver damage during virus-

induced hepatitis progression. FGL2 expression is required for maintaining the proinflammatory Ly6C^{high} MoMF phenotype by mediating IRF3, p65, and p38 phosphorylation, thereby forming a positive feedback loop of inflammatory accumulation in the liver.

ARTICLE HIGHLIGHTS

Research background

Viral fulminant hepatitis (VFH) is a devastating syndrome that pathologically caused by excessive activation of both innate and adaptive immunity. However, the extent of contribution of innate immunity in VFH is not well defined. Macrophage polarization have been implicated in host defense and the pathogenesis of various hepatic diseases. Fibrinogen-like protein 2 (FGL2) can be induced robustly and exclusively in macrophages in response to cytokines or viral infection. Exploring their roles in VFH can greatly improve our understanding of the disease and thus seek the therapeutic approaches.

Research motivation

Hepatic macrophages are attractive therapeutic targets because their functions can be inhibited or augmented to alter disease outcomes. A better understanding of their biological properties and immunologic function in liver homeostasis and pathology may pave the way for new diagnostic and therapeutic approaches for liver failure or other liver diseases.

Research objectives

To evaluate the role of Fgl2 in the reconstitution of hepatic macrophages during VFH progression by the VFH mouse model.

Research methods

Liver sections of liver failure patients and controls were immuno-stained for macrophages examination. Murine hepatitis virus strain 3 (MHV-3) was used to induce VFH experimental model in wild type and *Fgl2*-/- mice. Adoptive transfer or depletion of macrophages were employed to assess liver damage and hepatic macrophages alteration. Signal cascade induced by LPS or MHV-3 were detected in macrophages.

Research results

Infiltrated MoMFs is a major source of hepatic inflammation during VFH progression. Fgl2 expression on macrophages prompts and enhances the inflammatory phenotype of hepatic macrophages, which breaks the previous understanding that the mechanism of Fgl2 during VFH is generally considered to be procoagulant activity.

Research conclusions

We revealed for the first time that pro-inflammatory monocyte-derived macrophages (MoMFs) infiltration is critical event for hepatic inflammatory accumulation and subsequent liver damage during virus-induced hepatitis progression and Fgl2 is required for maintaining the pro-inflammatory hepatic macrophages phenotype.

Research perspectives

Macrophages are 'keystones' of liver architecture in both homeostasis and disease. The development of potential therapies highly depends on a fundamental knowledge about the mechanisms that trigger the polarization and control the fate of hepatic macrophages. We believe that a better understanding of the specific mechanisms underlying macrophages participation in diseases will definitely result in the increased efficacy of these therapies.

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LETTER TO THE EDITOR

Interplay between chronic hepatitis B and atherosclerosis: Innovative perspectives and theories

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Abstract

Elaboration of carotid atherosclerosis in the setting of hepatitis B virus (HBV) infection should emphasize the significance of extrahepatic manifestations of the infection pathogenesis. Diverse processes comprise the pathoevolution of HBV infection, rendering it a multi-systemic disease in its essence. Our work not only exemplified atherosclerosis as an often-underestimated contributor to the severity of HBV infection but has also highlighted the bidirectional relationship between the two. Therefore, it is suggested that HBV-induced inflammation is one of the root causes of atherosclerosis, which in turn has a consequent effect on the severity of the chronic infection disease state, creating a vicious cycle. Additionally, we coupled prior data with the current concepts of HBV infection to postulate intriguing perspectives and theories.

Key Words: Hepatitis B virus infection; Carotid atherosclerosis; Hepatitis B e antigen; Arterial inflammation; Perspectives and trends

Core Tip: Hepatitis B virus (HBV) infection is a multifaceted disease, with significant cardiovascular morbidity. Our innovative approach to this pathophysiologic

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relationship harbors several key ideas. First, HBV infection may carry a specific atherosclerosis distribution pattern, with predilection for carotid arteries. Second, we propose wider use of more sensitive inflammatory markers, such as high-sensitivity C-reactive protein and homocysteine. Third, macrophage phenotype function should be investigated, utilizing its potential role as an atherosclerosis biomarker in HBV infection and therapeutic target. Last but not least, we reason that statins should be exploited more in current practice, due to their favorable pleotropic effects.

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TO THE EDITOR

We read with interest the study of Riveiro-Barciela *et al*[1] which elucidates the possible interplay between hepatitis B virus (HBV) infection and carotid atherosclerosis. It has high-yield trial properties due to its large sample size and prospective method. Although its inclusion and exclusion criteria were precise and broad, as with any case-control study, there remains the possibility of bias as a consequence of inferring causation from statistically significant correlations which can be complicated by difficulty in determining the chronological order of exposure to HBV (*i.e.* the starting time of infection and latency).

The authors concluded that the presence of subclinical atherosclerosis and carotid plaques were more frequent in patients with HBV infection than in controls and that liver damage was an independent factor associated with subclinical atherosclerosis and carotid plaques, regardless of the presence of classical cardiovascular factors.

In general, we agree with the authors, since many of our patients render a similar atherosclerotic disease profile which cannot be attributed solely, *sui generis*, to the cardiovascular substrate. Therefore, their study's findings have the capacity not only to raise the index of suspicion of a practicing clinician but to optimize the established diagnostic framework of HBV patients in order to prevent atherosclerosis occurrence and complications.

Furthermore, the study implicates chronic HBV infection (*i.e.* the specific point of the naïve hepatitis B e antigen (HBeAg)-negative phase) as being an important atherosclerotic contributor. Conversely, a prior study by Tong *et al*[2] has concluded that HBV infection not only negatively correlates with C-reactive protein (CRP) levels but seems to not be associated with coronary atherosclerosis. Additionally, Kiechl *et al*[3] found no significant association between chronic hepatitis and the development of new carotid atherosclerotic plaques; although, they did not specify the type of hepatitis virus. Of course, these conflicting results have to be considered cautiously, as they originate from patients in different phases of the HBV infection and divergent research materials and methods. With all this said, it may be that the window of opportunity for early atherosclerosis detection and preemptive therapeutic intervention in HBV could represent the subpopulation of naïve and HBeAg-negative patients.

However, the general discrepancies in the conclusions of the aforementioned trials made us postulate some intriguing perspective theories. First, it may be that HBV infection harbors specific propensity towards anatomically different vascular structures, thereby affecting carotid arteries more often than coronary arteries. This notion is in concordance with the previously published data inferring that viruses have different sites of endothelial predilection[4]. Second, we may utilize other, more sophisticated inflammatory markers, namely high-sensitivity (hs-)CRP with or without homocysteine for optimal HBV patient stratification regarding atherosclerosis risk[5]. Third, the potential role of macrophage phenotype variation during HBV infection may be one of the crossroads between the processes of atherosclerosis and HBV infection[6]. Current cardiology investigations have revealed the significant role of macrophages, encompassing their local, endothelial, as well as systemic effects *via* T-helper lymphocytes and cytokine release modulation[7]. Having stated that, we

postulate that HBV infection may trigger macrophage phenotype alteration, rendering it to be a contributive precipitant of atherosclerotic disease as well as the crosslink point between the two diseases. Last but not least, the study of Riveiro-Barciela *et al*[1] may open the door for broader statin use, addressing two end goals concomitantly: Lowering the risk of cirrhosis and hepatocellular carcinoma in viral hepatitis patients [8], and engaging in the prevention and treatment of atherosclerosis and its complications.

We believe that prospects in this field should be diversified in the manner that one-size does not fit all. Upcoming trials and future viewpoints should render better comprehension of the delicate HBV pathodynamics from which implementation of optimized and specific therapy would be more feasible.

Our group envisions many possible pathways between HBV infection and atherosclerosis, *i.e*. cardiovascular diseases which may be potential targets for clinical management, and thus encourages future research work in this field.

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LETTER TO THE EDITOR

Is CA19-9 effective in predicting chemotherapeutic response in patients with synchronous liver metastases with colorectal cancer?

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Abstract

Evaluation of response to chemotherapy in colorectal cancer patients with synchronous liver metastases is important in terms of treatment management. In this Letter to the Editor, several issues in the article are discussed. For the comparison of carbohydrate antigen 19-9 (CA19-9) values referenced in the study, the patient group was not matched for cancer stage. Therefore, it may be more appropriate to select and compare CA19-9 values in patients with same-stage cancer.

Key Words: Colorectal cancer; Carbohydrate antigen 19-9; Liver metastasis of colorectal cancer; Synchronous liver metastasis; Chemotherapy; Metastatic colorectal cancer

Core Tip: It is important to evaluate synchronous liver metastases of colorectal cancer (CRC) and to determine the response to chemotherapy in patients. Based on findings from such, the optimal treatment method is selected for each patient. The scoring system described in the study, created through a combination of radiology and laboratory parameters, can guide treatment. However, we think that it would be more appropriate to discuss the results of this study in the context of other studies conducted with patients with stage IV CRC.

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TO THE EDITOR

Ma *et al*[1] recently published a retrospective study on the emerging role of a magnetic resonance imaging (MRI)-radiomics signature to detect the predictive efficiency of models for chemotherapeutic response in colorectal cancer (CRC) patients with synchronous liver metastasis (SLM) and avoid ineffective chemotherapy.

Carbohydrate antigen 19-9 (CA19-9) has been routinely studied in patients with CRC, and in the study by Ma *et al*[1] the measurement of CA19-9 was found to be significant between the disease non-response (non-DR) and disease response (DR) to chemotherapy groups (P = 0.045). The authors showed that CA19-9 Levels were higher in the DR (63.3%) group than in the non-DR group (43.4%). The authors reported that CA19-9 is a promising indicator for predicting response to chemotherapy, citing the study by Zhou *et al*[2]. However, the study design used by Zhou *et al*[2] had included patients with stage III CRC, while the study by Ma *et al*[1] focused on patients with stage IV CRC.

Although it is known that high CA19-9 Levels are a poor-prognosis factor in untreated stage IV CRC patients, routine measurement of CA19-9 in colon cancers is not recommended by the American Society of Clinical Oncology (ASCO) guidelines due to insufficient evidence[3,4]. As such, we believe that it would be more appropriate to discuss the results of the study by Ma *et al*[1] in the context of other studies conducted with stage IV CRC patients[4,5].

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