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EDITORIAL

Combination treatment of inflammatory bowel disease: Present status and future perspectives

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Abstract

The treatment of patients with inflammatory bowel disease (IBD), especially those with severe or refractory disease, represents an important challenge for the clinical gastroenterologist. It seems to be no exaggeration to say that in these patients, not only the scientific background of the gastroenterologist is tested, but also the abundance of "gifts" that he should possess (insight, intuition, determination, ability to take initiative, *etc.*) for the successful outcome of the treatment. In daily clinical practice, depending on the severity of the attack, IBD is treated with one or a combination of two or more pharmaceutical agents. These combinations include not only the first-line drugs (*e.g.*, mesalazine, corticosteroids, antibiotics, *etc*) but also second- and third-line drugs (immunosuppressants and biologic agents). It is a fact that despite the significant therapeutic advances there is still a significant percentage of patients who do not satisfactorily respond to the treatment applied. Therefore, a part of these patients are going to surgery. In recent years, several small-size clinical studies, reviews, and case reports have been published combining not only biological agents with other drugs (*e.g.*, immunosuppressants or corticosteroids) but also the combination of two biological agents simultaneously, especially in severe cases. In our opinion, it is at least a strange (and largely unexplained) fact that we often use combinations of drugs in a given patient although studies comparing the simultaneous administration of two or more drugs with monotherapy are very few. As mentioned above, there is a timid tendency in the literature to combine two biological agents in severe cases unresponsive to the applied treatment or patients with severe extraintestinal

manifestations. The appropriate dosage, the duration of the administration, the suitable timing for checking the clinical and laboratory outcome, as well as the treatment side-effects, should be the subject of intense clinical research shortly. In this editorial, we attempt to summarize the existing data regarding the already applied combination therapies and to humbly formulate thoughts and suggestions for the future application of the combination treatment of biological agents in a well-defined category of patients. We suggest that the application of biomarkers and artificial intelligence could help in establishing new forms of treatment using the available modern drugs in patients with IBD resistant to treatment.

Key Words: Biologics for immune-mediated conditions; Dual-targeted treatment; Combination treatment; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

Core Tip: During the last few years, the combination of two biological agents or a combination of a biological agent and another drug belonging to the category of so-called "small molecules" seems to be steadily gaining ground [dual biologic therapy (DBT)]. Even the combination of a biological agent with a drug belonging for example to the category of immunosuppressants is a therapeutic option that has been applied for several years [combination therapy (CT)]. Finally, in daily clinical practice, various combinations of so-called first-line drugs (mesalazine, corticosteroids, antibiotics, probiotics, *etc.*) are used with satisfactory results in most cases. DBT and CT currently find application in cases of patients resistant to treatment or patients with extraintestinal manifestations that do not respond satisfactorily to classical treatment. The existing data, although encouraging, are not sufficient in terms of the number of patients included so far. The safety of this emerging kind of treatment is another point of interest. Finally, there is a need to carry out more studies regarding this interesting field of research.

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INTRODUCTION

It is known that biological agents, including small molecule drugs, alone or in combination with immunomodulatory drugs, are currently the recommended treatment in cases of moderate or severe inflammatory bowel disease (IBD)[1,2].

However, many patients, some of whom may have concurrently extraintestinal manifestations do not respond to treatment, making the treatment particularly complicated[3]. In recent years, the growth of our pharmaceutical arsenal has decisively influenced the way by which our IBD patients are treated. Certain characteristics of the disease in today's era, such as resistance to medication and the long duration of the disease may have an unsatisfactory therapeutic result. On the other hand, although the available drugs are effective even when they are given alone some patients do not respond favorably to treatment. Also, drugs that have a favorable effect on intestinal disease may not be effective in extraintestinal manifestations. The above assumption is also presumed from the fact that in the era of biological factors that we are going through, the rates of surgical interventions seem to be similar to those of previous years.

There are many inflammatory pathways involved in the pathogenesis of IBD as well as many cytokines involved in the inflammatory cascade. If we succeed in interrupting this cascade not only in one but in more places we expect to achieve a better clinical outcome. For example, tofacitinib improves rheumatoid arthritis and IBD because it inhibits inflammatory factors that contribute to both gut and joint inflammation[4]. It is therefore reasonable to hypothesize that administration of a combination of biological agents or a biological agent with an immunosuppressive or other pharmaceutical agent may increase the proportion of patients who respond to treatment.

We know that patients treated with biological agents may not respond to the initial treatment (primary non-response) or lose the good therapeutic effect at a later stage of the disease (secondary loss of effect). Anti-tumor necrosis factor (TNF) biological agents are monoclonal antibodies that suppress inflammation by binding to TNF-α[5]. Ustekinumab works by inhibiting the action of the pro-inflammatory cytokines Interleukin-12 and -23. Vedolizumab works by binding to the α4β7 integrin, resulting in an inability of T cells to infiltrate tissues and exacerbate inflammation. Tofacitinib is a small molecule that inhibits Janus kinase. The combination of these drugs, each of which has a different mechanism of action, will result in the inhibition of different inflammatory pathways. In this way and acting synergistically, these drugs can more effectively reduce the degree of the inflammatory process and improve the clinical and laboratory parameters of the patients.

During the last few years, several clinical studies and systematic reviews have been published describing the results of the combined use of biological agents in patients with IBD and patients suffering from severe rheumatological or dermatological diseases[6]. In this editorial, the results of the most important studies (results in patient series of clinical

trials, reviews, meta-analyses, and cases of interest) in which different drug combinations were used are listed. The results and side effects of the combinations are analyzed, with a simultaneous effort to highlight the most effective combinations in daily clinical practice.

A literature search on electronic databases such as PubMed, Medline, and Cochrane CENTRAL databases was performed to identify relevant articles. Keywords included biologics for immune-mediated conditions along with the terms "dual," and "combination." Case reports, case series, randomized controlled trials, systematic reviews, and meta-analyses were included.

RESULTS

Dual biological therapy

Dual biologic therapy (DBT) is a term that refers to the simultaneous administration of two biological agents or a biological and a micromolecular agent in patients with IBD, while the term Combination Therapy (CT) refers to combination of other drugs, mainly immunosuppressants with biological agents. These therapeutic approaches have already been applied to treatment-resistant IBD patients or patients with inactive disease but with difficult-to-treat extraintestinal manifestations[7,8]. Existing data support that DBT is a safe option for IBD patients who have failed treatments with single biologic agents as well as in patients with difficult-to-treat extraintestinal manifestations[9].

So far the most used combinations involve the concurrent administration of anti-TNF agents with vedolizumab or the concurrent administration of ustekinumab with vedolizumab. The combination of these agents emerged based on the satisfactory safety profile they present, as well as on the basis of the satisfactory degree of efficacy when administered as monotherapy[10-13].

DBT trials: A small number of clinical trials regarding the effectiveness and safety of DBT appeared in the literature. These studies are shown in Table 1.

In the first randomized trial published in 2007, the authors studied the safety and efficacy of the combination of natalizumab and infliximab (IFX) in patients with Crohn's disease (CD) unresponsive to IFX therapy[14]. A total of 52 patients received the combination of natalizumab and IFX and 27 patients received IFX and placebo. Regarding safety, adverse events occurred in 92% of patients receiving IFX and natalizumab and in all patients in the placebo group. The most frequently observed side effects were related to the occurrence of headache. No differences were observed in the incidence of infections between the two groups (27% *vs* 30%). After the first 10 wk, no serious side effects (infection, cancer, death) were observed in patients who continued to receive the combination of IFX and natalizumab. Regarding the clinical effectiveness, patients belonging to the group of the combination of biological agents showed better results than the placebo group, but the differences were not statistically significant. Despite the satisfactory results, this combination should be avoided due to the side effects of natalizumab (risk of multifocal leukoencephalopathy).

In a retrospective study, Yang *et al*[15] evaluated the safety and efficacy of DBT in 22 patients with refractory CD by administering seven different combinations of six biological agents that included an anti-TNF-α agent (IFX, adalimumab, golimumab, and certolizumab pegol), in combination with vedolizumab or ustekinumab. Endoscopic improvement and endoscopic remission were noticed in 43% and 26% of trials, respectively, and clinical response in 50%. Clinical improvement in perianal fistulas was also observed. Adverse events were observed in 13% of trials[15].

Two other retrospective studies published in 2020 evaluated DBT in patients with CD and ulcerative colitis (UC). The first of these included 16 patients (11 with CD and 5 with UC). Seven patients received DBT for unresponsive disease and 9 patients received DBT because of persistent extraintestinal manifestations despite remission of intestinal disease. Patients were treated with anti-TNF agents in combination with vedolizumab or ustekinumab while 2 patients received combined treatment with vedolizumab and ustekinumab for 8 wk. Clinical improvement of both intestinal and extra-intestinal manifestations was observed in all patients. Adverse effects were observed in 3 patients, all non-serious[11].

In the second study, 50 IBD patients (32 with CD and 18 with UC) received CT. The majority of patients received concomitant immunosuppressants or corticosteroids. A total of 29 of the 50 patients received combined biologic therapy. Adverse effects were reported in 26% of patients the majority of which were infectious[12].

Vedolizumab represents an effective and safe biological agent in the treatment of IBD patients during pregnancy, in elderly patients, in patients who have undergone surgery in the past, as well as in patients with a previous history of malignancy. Due to the advantages of the drug, mainly in the area of safety, vedolizumab is currently the main biological agent for the application of CT with another biological or small molecule agent[16,17]. The combination of vedolizumab with ustekinumab appears to be the most promising based on data from retrospective studies, descriptions of patient series, and individual cases. This combination should be a major area of future research since this regimen may be effective in both CD and UC patients.

Upadacitinib is a second-generation selective Janus kinase inhibitor targeting the JAK1 enzyme approved for the treatment of severe rheumatoid arthritis that is unresponsive to first-line therapy. Ten CD patients refractory to medical therapy were treated with a combination of Upadacitinib and Ustekinumab. Patients were followed-up for 10 months. The median number of prior biologic treatment exposures was 4. Indications for the use of DBT were active CD (6 patients), extraintestinal manifestations (2 patients), and both active CD and extraintestinal manifestations (2 patients). The results showed that 5 out of 6 patients with active CD achieved clinical remission and 2 patients with severe arthritis showed significant clinical improvement. Side-effects included mild respiratory symptoms and nausea. It seems that DBT with Upadacitinib and Ustekinumab may be effective and safe in refractory CD as well as for patients with extraintestinal manifestations[18].

Table 1 Results of clinical trials with dual biologic therapy in patients with active inflammatory bowel disease

Ref.	No. of patients/disease	Kind of DBT	Efficacy	Side-effects
Sands *et al* [14], 2007	79 (two arms: 52 and 27 respectively) CD	IFX + Natali-zumab *vs* IFX + placebo	Better results in DBT but not significant	Headache, infections (27%), nausea, nasopharyngitis
Privitera *et al*[11], 2020	16 (11 with CD & 5 with UC)	anti-TNF + VDZ or UST or VDZ & UST for 8 wk	Clinical improvement (intestinal and extraintestinal manifestations): In all patients	In 3 patients, all non-serious
Glassner *et al*[12], 2020	50 IBD patients (32 CD, 18 UC)	29 out of 50 patients received DBT	DBT: More patients in clinical and endoscopic remission at follow-up *vs* baseline	In 26% of pts. Infections: In patients on DBT. Lower risk in those not on a concomitant immunomodulator
Yang *et al* [15], 2020	22 patients with CD with 24 therapeutic trials of DBT	Six biologic agents were used in: Anti-TNF + UST or VDZ	Endoscopic improvement: In 43% of trials. Endoscopic remission: 26%. Clinical response: 50%. Clinical remission: 41%	Similar rates of adverse events (13% of trials)
Kwapisz *et al*[13], 2021	15 (14 CD, 1 UC)	Various biologics VDZ + anti-TNF/UST; UST + anti-TNF/VDZ	DBT may be effective. Anti-TNF or VDZ plus UST were most effective	DBT may be safe
Feagan *et al* [19], 2023	Severe UC	GUS + GOL (71) *vs* GUS alone (72) *vs* GOL alone (71 pts)	At week 12, 83% of DBT patients had clinical response *vs* 61% and 75% on GOL and GUS monotherapy, respectively	At week 50, 63%, 76% and 65% of patients experienced at least one side-effect (infections, fever, nasopharyngitis, neutropenia
Miyatani *et al*[18], 2024	CD	UPA + UST	5/6 patients, achi-evedremission. Two with severe arthritis: Signifi-cant improvement	Mild respiratory symptoms and nausea

CD: Crohn's disease; GOL: Golimumab; GUS: Guselkumab, IBD: Inflammatory bowel disease; IFX: Infliximab; TNF: Tumor necrosis factor; UC: Ulcerative colitis; UST: Ustekinumab; UPA: Upadacitinib; VDZ: Vedolizumab; DBT: Dual biologic therapy.

In a randomized, double-blind, controlled trial[19], Feagan *et al*[19] investigated whether DBT with Guselkumab (antagonist of the p19 subunit of IL-23) and Golimumab (TNF-a inhibitor) is superior to monotherapy with these two drugs separately administered to patients with moderately to severely active UC. DBT consisted of sc golimumab 200 mg at week 0, sc golimumab 100 mg at weeks 2, 6, and 10, and iv guselkumab 200 mg at weeks 0, 4, and 8, followed by sc monotherapy with guselkumab 100 mg every 8 wk for 32 wk, whereas golimumab monotherapy consisted of sc golimumab 200 mg at week 0 followed by sc golimumab 100 mg at week 2 and every 4 wk thereafter for 34 wk, and guselkumab monotherapy consisted of iv guselkumab 200 mg at weeks 0, 4 and 8, followed by scguselkumab 100 mg every 8 wk thereafter for 32 wk. Of the 214 patients who were finally included, 71 patients received DBT, 72 patients received golimumab monotherapy, and 71 patients received guselkumab monotherapy. Results showed that at week 12, 83% of DBT patients showed a clinical response compared to 61% of patients in the golimumab monotherapy group, and 75% of the guselkumab monotherapy group. At week 50, 63%, 76%, and 65% of patients in the three groups reported at least one side effect such as respiratory infections, nasopharyngitis, neutropenia, and fever. It therefore appears that DBT with guselkumab and golimumab is superior to treatment with one agent alone.

Safety of DBT: An element of DBT that is equally important as its effectiveness concerns the degree of safety provided. Safety has been an important element of the available studies with several of them claiming to have found no serious adverse effects, with most of which referred to an increased risk of infections[15]. In a retrospective observational study, the efficacy and safety of DBT with the combination of two biological agents or the combination of one biological agent with a small molecule was studied. The results showed clinical and endoscopic improvement in 50% of patients with parallel improvement of extraintestinal manifestations. However, a significant percentage of adverse effects (42%) and an increased risk of infections were observed, which necessitated hospitalization in 10%[20].

According to Privitera *et al*[11] adverse effects with DBT with ustekinumab and vedolizumab were observed in 13% to 30% of patients with infections being the most common side effect[11]. The results of a recent systematic review and meta-analysis agree with this percentage[8]. It therefore appears that DBT with vedolizumab and ustekinumab has a tolerable rate of side effects apparently as a result of the low rate of side effects shown by each of these drugs individually. It is recommended that patients be systematically monitored for any unwanted effects, mainly infections and even infections due to rare causes. The exact magnitude of the risk of side effects is expected to be determined in future multicenter studies.

Combination therapy of IBD

We currently distinguish two forms of CT of anti-TNF-α agents and immunosuppressants. In the first combination, called de novo, the combination of the two drugs is done right from the beginning, *i.e.* from the start of the treatment. The purpose of co-administration from the outset is to prevent the formation of antibodies against the biological agent. In the second CT, the so-called "selective", the immunosuppressant is added quite later and only in patients who show a secondary loss of response during anti-TNF-α monotherapy due to the development of antibodies against the biological

agent[21].

It is known that the combination of IFX and thiopurines is superior to monotherapy in inducing and maintaining remission in IBD patients both at the clinical level and in the generation of antibodies against IFX, but at the cost of an increased risk of infections and neoplasms (*e.g.* lymphoproliferative disease). This risk could be partially avoided by reducing the dose, but this needs to be proven in the future. Even the combined treatment could be used for a short period (*e.g.* one year) since it is known that antibodies against the biological agent usually develop during the first months of treatment.

Several studies have proven the truth of the above. In a recently published network meta-analysis and systematic review, the authors evaluated the efficacy and safety of CT with IFX and azathioprine versus IFX monotherapy in CD patients. The study included 15 Randomized clinical trials (RCT) with a total of 1586 CD patients. The results showed that both therapeutic strategies are comparable in terms of their efficacy and safety since no differences were observed in the induction and maintenance of remission between the two combinations. No treatment was significantly safer than the others[22].

Clincal trials concerning CT in IBD patients are shown in Table 2.

CT of IFX with immunosuppressants: A randomized, double-blind study evaluated the efficacy and safety of combined administration of IFX with azathioprine (AZA), compared with AZA or IFX alone in patients with moderate to severe UC. It was found that patients treated with IFX with AZA had a greater rate of disease remission as well as higher rates of mucosal healing compared to patients treated with AZA alone or the biologic agent alone[23].

In 2015 Colombel *et al*[24] published the results of the post hoc analysis of the SONIC trial. The results of the study showed that the CT of IFX with AZA was more effective compared to monotherapy with AZA or IFX, suggesting that mucosal healing can be achieved with CT in a high percentage of patients with early CD[24].

Regarding the dose of AZA in IBD patients in whom disease remission was achieved with CT of IFX with AZA, Roblin *et al*[25] observed that reducing the dose of AZA but not stopping it in patients receiving CT (IFX with AZA) has the same efficacy as the efficacy of continuing full-dose AZA[25].

The anti-TNF agent IFX has also been used as CT with methotrexate. In a double-blind, placebo-controlled study lasting 50 wk, in which IFX was administered with Methotrexate or IFX alone, it was shown that the combination of the two drugs, although safe, did not significantly differ in efficacy from the administration of IFX alone[26].

It is a fact that there is widespread reluctance among gastroenterologists to administer immunosuppressants or biologic agents to elderly IBD patients because of the increased potential for side effects. In a relevant study, Singh *et al* [27] evaluated the effect of age in CD patients older than 60 years, in terms of efficacy and side effects over 2 years. Patients were randomized to receive early combined immunosuppression (173 patients) or conventional management (138 patients). During the 24-month follow-up period, 10% of elderly patients developed CD-related complications (early combined immunosuppression 6.4% versus conventional treatment 14.5%). No difference was found regarding the safety and efficacy of early combined immunosuppression compared with conventional management in both elderly and younger patients. Therefore, early combined immunosuppression is indicated as a therapeutic option in selected elderly CD patients who do not show a satisfactory therapeutic response[27].

In the SONIC and UC-success study, the combination of IFX and AZA was superior to treatment with IFX alone in both UC and CD patients[28]. Therefore, the combined administration of IFX and AZA in patients at low risk of toxicity and patients with limited therapeutic options is expected to provide significant help under the terms and conditions mentioned above.

Louis *et al*[29] compared the relapse rate and duration of remission over two years in the group of CD patients who continued DCT therapy with IFX plus AZA (n = 67) and the group of patients who discontinued treatment with IFX while maintaining AZA (n = 71), as well as the group that maintained IFX therapy but discontinued AZA (n = 69). A total of 39 patients relapsed (12% of the DBT group, 35% of the IFX discontinuation group, and 9% of the AZA discontinuation group). The 2-year relapse rates were 14% in the combination group, 36% in the IFX withdrawal group, and 10% in the immunosuppressant withdrawal group. A total of 31 serious adverse events were observed in 20 patients, with no difference between groups. The most common serious side effects were infections. No death or malignancy occurred. It therefore appears that in CD patients in remission on CT with IFX and AZA, discontinuation of IFX should only be done on a case-by-case basis while withdrawal of AZA is the preferred de-escalation strategy[29].

Roblin *et al*[30] compared two therapeutic strategies, namely changing the anti-TNF agent to another, or adding an immunosuppressant to the initial treatment while maintaining the same anti-TNF agent in 90 patients with IBD in clinical relapse who presented undetectable anti-TNF trough levels and antidrug antibodies. The rate of clinical failure and occurrence of adverse pharmacokinetic curves were higher in monotherapy compared to CT. At a follow-up of 24 months, the survival rates without clinical failure or adverse pharmacokinetics of the biological agents were 22% *vs* 77% and 22% *vs* 78% (monotherapy *vs* CT)[30]. The authors recommend the use of CT after switching to the anti-TNF agent to have favorable clinical outcomes.

A practical question that arises after the successful administration of CT in patients with IBD concerns the duration of maintenance therapy. Lambrescak *et al*[31] investigated the likelihood of disease recurrence two years after achieving remission with CT in 139 patients with a median follow-up of 18.9 months. They noticed that in the 26 relapsed cases shorter duration of CT was not associated with an increased risk of treatment failure. The results do not support the view of continuing CT for more than 12 months after achieving clinical remission in IBD patients[31].

Regarding the route of administration of IFX (subcutaneous or intravenous administration) D'Haens *et al*[30] found that the pharmacokinetics, efficacy, and immunogenicity of the two routes of administration were comparable in the group of patients who underwent monotherapy with sc IFX and DBT in biologic-naïve IBD patients[32].

Table 2 Results of clinical trials with combination therapy in patients with active inflammatory bowel disease

Ref.	Disease	Kind of CT	Efficacy
Colombel *et al* [24], 2015	Severe UC	IFX + AZA *vs* IFX alone *vs* AZA alone	CT was more effective compared to monotherapy with AZA or IFX. High rate of mucosal healing with CT
Feagan *et al* [26], 2014	CD	IFX + MTX *vs* IFX alone *vs* MTX alone	No significant differences. Safe combination
Louis *et al*[29], 2023	CD	IFX + AZA *vs* AZA alone *vs* IFX alone	Relapse rate: 12% in the DBT group compared to 35% (IFX group) and 9% in the AZA group. Most frequent side-effects: Infections
Roblin *et al*[30], 2020	IBD 90 patients	Therapeutic strategies: Change of anti-TNF agent to another or adding immunosuppressant	The rate of clinical failure and occurrence of adverse pharmacokinetic curves were higher in monotherapy compared to CT. Use of CT after switching to the anti-TNF agent is recommended
Matsumoto *et al* [34], 2016	CD	Monotherapy *vs* combination group (ADA + AZA *vs* ADA alone)	Remission rate at week 26 did not differ between the two groups. Thus, combination of ADA with AZA offers no benefit compared to ADA alone
Christensen *et al* [36], 2019	9 patients with CD and 11 with UC	VDZ + calcineurin inhibitors	CT of VDZ with calcineurin inhibitors is a safe and effective combination to induce remission in IBD
Sands *et al*[37], 2019	CD	VDZ + CS *vs* VDZ alone *vs* CS alone	CT: Higher rates of clinical remission compared to the other groups. Similar adverse events

ADA: Adalimumab; anti-TNF: Anti-tumor necrosis factor; AZA: Azathioprine; CD: Crohn's disease; CT: Combination treatment; CS: Corticosteroids; IBD: Inflammatory bowel disease; IFX: Infliximab; UC: Ulcerative colitis; VDZ: Vedolizumab; DBT: Dual biologic therapy.

CT of adalimumab with immunosuppressants: Regarding the combined administration of adalimumab with immunosuppressants (thiopurines) as a maintenance treatment, according to the data of an earlier study, the continuation of the administration of thiopurines for a period longer than 6 months does not offer a substantial benefit compared to monotherapy with adalimumab[33].

In contrast to IFX with AZA CT, the combination of adalimumab with AZA appears to offer no additional benefit compared to adalimumab alone. In an open-label prospective study Matsumoto *et al*[34], evaluated the efficacy of adalimumab with or without AZA in 176 patients with active CD who had not previously been treated with biological agents for 52 wk. It was found that the remission rate at week 26 did not differ between the two groups although the endoscopic improvement rate at week 26 was significantly higher in the CT group compared to the monotherapy group. Furthermore, the clinical efficacy of AZA with adalimumab CT at week 26 did not differ from that of adalimumab monotherapy[34].

Vedolizumab with calcineurin inhibitors: Vedolizumab is a potentially effective maintenance regimen after salvage therapy achieved with calcineurin inhibitors in acute severe UC and DTT is recommended as a potential option in these patients[35]. The combination of vedolizumab with calcineurin inhibitors in patients with UC or CD has been used for at least six years. Christensen *et al*[36] published the results of CT of vedolizumab plus calcineurin inhibitors in 20 patients with IBD (9 with CD and 11 with UC) for 12 months after starting treatment with vedolizumab. In the first 12 wk of treatment, 44% of CD patients and 55% of UC patients achieved clinical remission without using corticosteroids. After one year of treatment, 33% of CD patients and 45% of UC patients were in clinical remission without steroids. The 3 serious adverse events that occurred were related to the calcineurin inhibitors and not to the biological agent. These results, although in a small number of patients, suggest that CT of vedolizumab with calcineurin inhibitors is a safe and effective combination in terms of inducing and maintaining remission in patients with IBD for at least one year[36].

Vedolizumab with corticosteroids: Sands *et al*[37] evaluated the efficacy and safety of vedolizumab co-administered with corticosteroids as induction therapy in patients with moderate-to-severe active CD. The data of this retrospective study evaluated the results of induction therapy after 6 and 10 wk of the GEMINI 2 and GEMINI 3 studies. The results showed that the combination of vedolizumab with corticosteroids resulted in higher rates of clinical remission compared to the CT of corticosteroids with placebo, as well as compared to the vedolizumab-only group. The combination of vedolizumab and corticosteroids achieved significantly higher rates of clinical response compared to the administration of corticosteroids compared to patients who received vedolizumab alone. Adverse event rates were similar between groups. It thus appears that vedolizumab in combination with corticosteroids improves remission or clinical response rates in patients with moderately to severely active CD[37].

Combination treatment with drugs used as a first-line therapy of patients with IBD

In daily clinical practice, combinations of two, three, or even more drugs are often used as the first line of treatment for patients with IBD depending on the severity and extent of the disease (*e.g.*, mesalazine, corticosteroids, antibiotics, probiotics, *etc*). For the combinations of these drugs, the existing data seem to be relatively insufficient. The most important of the existing studies regarding combinations of these drugs are listed below.

CT with antibiotics in severe UC: It is generally accepted that the administration of antibiotics in UC flares lacks a favorable clinical outcome. Their administration is required only in cases in which there is "serious suspicion of septic complications". This aphorism, however, lacks practical significance since in the event of a serious relapse, both stool and blood cultures will be available after at least three days during which the patient experiences symptoms characterized by bloody diarrhea with fever, anorexia, vomiting, abdominal pain, *etc.*, and on the other hand because it is not certain that any microbial sepsis will necessarily be demonstrated in the culture. For this reason, the vast majority of clinical gastroenterologists dealing with the treatment of IBD worldwide prefer, in case of severe UC, the "blind" administration of a combination of antibiotics (mainly metronidazole and ciprofloxacin) for at least five days, alongside the administration of the intense treatment regimen.

Recently, three clinical studies have been published regarding the administration of combination antibiotics in patients with active UC. The first of these evaluated the administration of a combination of three oral antibiotics (500 mg amoxicillin, 500 mg tetracycline, and 250 mg metronidazole three times daily) in 30 patients with active UC-resistant or dependent on corticoids. The results showed that 19 of 30 steroid-resistant patients and 47 of 64 steroid-dependent patients showed a clinical response at 2 wk. After 3 and 12 months the percentages of patients with clinical remission in the first group were 60% and 66.6% respectively and in the second group 56.3% and 51.6% respectively. Ten percent of the first group and 6.3% of the second group underwent colectomy. This study, although lacking a control group, supports that the combined administration of these three antibiotics is effective and safe in patients with active steroid-resistant or steroid-dependent UC[38]. It is worth mentioning that these patients were given a combination of antibiotics used in *Helicobacter pylori* eradication therapy. The paper does not mention this parameter or the status of any *Helicobacter pylori* infection.

The possibility that the favorable effect of the administration of these three antibiotics was due to a long-term change in the intestinal flora of UC patients was investigated in a subsequent multicenter, randomized, double-blind, placebo-controlled study. For this purpose, mucosal samples were taken from 20 patients at the beginning of the treatment and 3 months after its completion to detect terminal restriction fragment length polymorphism in mucosa-associated bacterial components. The researchers found changes in mucosa-associated bacterial components in 10 of 12 patients in the treatment group and none of 8 in the placebo group. These changes persisted for more than three months after completion of treatment, suggesting that treatment with these antibiotics results in long-term changes in the microbiota of patients with UC that may contribute to the favorable therapeutic outcome[39].

The second study evaluated the combined administration of two drugs (ceftriaxone and metronidazole or placebo) as adjunctive therapy in 50 patients with severe UC exacerbation. The authors found that the addition of the two antibiotics in addition to standard care, did not improve outcomes in patients with severe UC exacerbation. However, it should be taken into account that the evaluation of the results was done on the third day of treatment and that the number of cases of fulminant colitis was twice as high in the antibiotic group, which objectively implies that the exacerbation was more severe in the patients in the antibiotic group[40].

In the third study, Rhodes *et al*[41] investigated the efficacy and safety of a combination of antibiotics (ciprofloxacin 500 mg *bd*, plus doxycyclin 100 mg *bd*, plus hydroxychloroquine 200 mg *tds* for 4 wk, followed by doxycycline 100 mg *bd* and hydroxychloroquine 2 mg *tds* for 20 wk in 39 patients with CD) versus budesonide (9 mg peros for 8 wk, 6 mg/d for 2 wks and 3 mg/d for 2 wk in 39 patients with CD). Results were promising with 9/24 patients receiving antibiotics/hydroxychloroquine per protocol maintained in remission by week 24. The overall results with the antibiotic/hydroxychloroquine combination were not impressive, but long-term remission was observed in some patients, which warrants further studies. Withdrawals from the study due to adverse events were observed in 15 patients who received the antibiotic combination and in 6 of those who received budesonide[41].

CT of corticosteroids with mesalazine in severe UC: In a randomized, controlled, investigator-blinded, clinical trial in patients with severe UC exacerbation, 149 patients were treated with corticosteroids alone (73 patients) or corticosteroids plus mesalazine 4 g/d (76 patients). The results showed that 72.6% of patients who received corticosteroids and mesalamine responded to treatment compared to 76.3% of patients treated with corticosteroids alone. The need for administration of biological agents was numerically lower in the group of patients who received corticoids and mesalazine, but the differences did not reach statistical significance. It therefore appears that the combination of mesalamine with corticosteroids does not provide a statistically greater benefit than corticosteroids alone in patients with severe UC exacerbation[42].

CT of oral and rectal mesalazinein UC: In patients with mild to moderate UC, the combined administration of 4 g/d oral and 1 g rectal mesalazine for 8 wk resulted in significantly higher remission rates compared with 4 g/d oral mesalazine and placebo from the rectum (64% *vs* 43%, respectively, PINCE study). All the indices (*e.g.* disease activity index, speed of bleeding elimination, mucosal healing, and quality of life level) were significantly improved in the combined treatment group[43].

CT of antibiotics with vedolizumab in pouchitis: Pouchitis is a major complication occurring in 50% of UC patients who have undergone Ileo Anal Pouch Anastomosis (IAPA). In 20% of patients with pouchitis, the disease becomes chronic. The treatment of this complication presents significant difficulties with high failure rates in several cases. In a recent RCT, Travis *et al*[44] evaluated the effect of vedolizumab (300 mg iv as a loading dose and every 8 wk thereafter) *vs* placebo in 102 patients with chronic pouchitis, while ciprofloxacin administration was maintained in both groups for the first 4 wk. The results showed significant superiority of the combined administration of vedolizumab and ciprofloxacin compared to ciprofloxacin and placebo (remission rate at week 14 31% *vs* 10%). The data regarding the adverse effects of the drugs were also of interest. Serious adverse events occurred in 6% of the vedolizumab group and 8% of patients in the placebo

group[44].

CT of adalimumab and ciprofloxacine in perianal fistula: The combined administration of adalimumab 40 mg every other week with ciprofloxacin 500 mg or placebo twice daily for 12 wk was significantly superior to monotherapy with adalimumab to achieve fistula closure in CD. In a randomized, double-blind, placebo-controlled trial, 76 patients with CD and active perianal fistula were enrolled. At 12 wk the degree of clinical response, reduction in Crohn's Disease activity index (CDAI), and increase in quality of life, were significantly superior in the group of patients who received adalimumab plus ciprofloxacin CT. No differences were observed regarding the rate of side effects. However, the favorable effect was not maintained after discontinuation of the antibiotic[45].

SYSTEMATIC REVIEWS AND METAANALYSES

So far, 5 systematic reviews with or no meta-analyses have been published investigating the efficacy and safety of DBT in patients with IBD. A meta-analysis of 7 studies with a total of 18 patients under DBT (vedolizumab with anti-TNF or ustekinumab) found that all study patients (100%) achieved clinical improvement while 93% showed endoscopic improvement. No significant adverse effects were observed during the 14-month follow-up[46]. Another meta-analysis evaluated the safety and efficacy of DBT and CT with a small molecule agent in patients with refractory IBD (anti-TNF with Vedolizumab, anti-TNF with Ustekinumab, and Ustekinumab with Vedolizumab). A total of 279 patients with refractory IBD and/or extraintestinal manifestations participated. The main indications for DBT administration were drug-resistant disease (81%) and concomitant extraintestinal manifestations of rheumatologic disease (12%). After a median follow-up of 32 wk, the results showed that 59% of patients achieved clinical remission and 34% endoscopic remission, while 12% required surgery. Serious side effects (mainly infections) occurred in 6.5%. Of interest was the fact that the success rate was higher in patients who were given DBT because of treatment-resistant extraintestinal manifestations. Both of these systematic reviews conclude that DBT is a satisfactory treatment option in specialized centers in selected patients with refractory disease or patients with extraintestinal manifestations not controlled by a single biological agent[8].

In a meta-analysis published in the same as the previous year (2022), the authors evaluated the safety and efficacy of the administration of two biological agents or one biological agent and a small molecule (vedolizumab plus anti-TNF-α (56 patients) or vedolizumab plus tofacitinib (57 patients). A total of 13 studies (mostly observational) involving 266 patients with 7 different combinations were included. Median follow-up ranged from 16 to 68 wk. The rate of adverse events for the combination of vedolizumab plus anti-TNF-α was 9.6% while for the combination of vedolizumab plus tofacitinib, the rate of side effects was 1%! The results of this meta-analysis also confirm that DBT is generally safe and effective[47].

In a recent systematic review, the authors analyzed the results of 29 studies in 288 patients with IBD who were given DBT for incompletely responding or non-responding. These patients were given a combination of anti-TNF plus anti-integrin (14 studies, 113 patients), vedolizumab plus ustekinumab (12 studies, 55 patients), vedolizumab plus tofacitinib (9 studies, 68 patients), anti-TNF plus tofacitinib (5 studies, 24 patients), anti-TNF plus ustekinumab (6 studies, 18 patients), and ustekinumab plus tofacitinib (3 studies, 13 patients). Again the authors concluded that DBT administration is a promising therapeutic approach for patients with partial or no response to targeted monotherapy[48].

Finally in a very recently published systematic review the authors analyzed 13 clinical trials evaluating eight biologic agents in patients with CD. Among the biologic agents evaluated, upadacitinib, vedolizumab, adalimumab, guselkumab, mirikizumab, ustekinumab and risankizumab showed statistically significant efficacy concerning various clinical and laboratory parameters (including biomarkers, histology, endoscopy and quality-of-life). Regarding safety it was noticed that all biologic agents were well tolerated with a good safety profile. The authors of this systematic review conclude that DBT could be considered as an effective and safe therapeutic modality for patients with active CD non-responding to conventional treatment[49].

CASE REPORTS AND CASE SERIES

Many case reports or case series have been published regarding the use of DBT in patients with resistant IBD. The majority of these descriptions focus on the use of an anti-TNF-α in combination with vedolizumab. A case series study included 10 patients (6 with UC and 4 with CD)[50]. The authors concluded that CT with vedolizumab and IFX or vedolizumab and adalimumab is probably a safe long-term regimen in patients with refractory CD. The study by Biscaglia *et al*[51] found that the administration of DBT (ustekinumab and vedolizumab in two patients with IBD resulted in improvement of intestinal disease and extraintestinal manifestations, while no adverse events were reported during the two-year follow-up under DBT treatment[51]. In another series of cases in which vedolizumab and other biological agents were administered for 5-37 months, the authors achieved clinical remission and improvement of extraintestinal manifestations. A small percentage of infections were observed, which were, however, not serious[52]. Bethge *et al*[53]. described a patient with enteropathic seronegative spondyloarthritis and refractory UC who eventually underwent IAPA. In this patient with refractory pouchitis, the combination of vedolizumab and etanercept resulted in endoscopic and histological remission with complete resolution of joint symptoms without significant adverse effects[53]. Roblin *et al*[54] described a case of a patient with severe, treatment-resistant UC and human leukocyte antigens-B27 positive

spondyloarthropathy treated with vedolizumab[54]. The patient responded satisfactorily to the addition of golimumab to the regimen. In the long term, both UC and spondyloarthropathy were maintained in remission after one year of CT with vedolizumab and golimumab. Liu *et al*[55] described a case of a young patient with ileocolic CD who, after 10 months of treatment with a combination of ustekinumab and vedolizumab, achieved mucosal healing for the first time after 13 years of persistent disease. During the six-month combined administration of the two biological agents, no significant side effects were found[55]. Huff-Hardy *et al*[56] combined vedolizumab with ustekinumab in a patient with refractory CD. The patient (female, aged 22 years) with severe, stenotic, fistula refractory to treatment showed significant improvement in perianal disease after 8 weeks of DBT while achieving deep remission after 1 year of treatment[56]. Finally, a recent multicenter study from Finland analyzed data from 16 patients (15 with CD) treated with a combination of two biologic agents. The DBT combination used in most patients was adalimumab plus ustekinumab with a median follow-up of nine months. Seven patients (32%) were in remission at the end of follow-up. In all centers from which data were collected, DBT reduced the need for corticosteroids. The majority of patients who achieved a response to DBT were treated with a combination of adalimumab and ustekinumab (56%). At the end of the follow-up, all nine (41%) DBT responders continued treatment. Infections occurred in three patients (19%). The experience of using DBT in this small number of patients is encouraging[57].

Summary of the results

A summary of the results of the studies, the results of which in the authors' opinion are valid and clinically applicable, is listed below.

Dual therapy (combination of two biological agents): Privitera *et al*[11], in a retrospective study of 16 patients with active IBD and/or patients with severe extraintestinal manifestations, used dual therapy (DT) consisting of a combination of vedolizumab + ustekinumab or vedolizumab + adalimumab. Clinical improvement of intestinal disease and/or extraintestinal manifestations was observed in all patients treated with DT without serious adverse events.

Kwapisz *et al*[13] in 14 patients with CD and 1 patient with UC used a CT consisting of two biological agents: Vedolizumab plus anti-TNF agent (8 patients), vedolizumab plus ustekinumab (5 patients) and ustekinumab plus anti-TNF- α agent (2 patients). Symptomatic improvement was noticed in 73%. Moreover, 67% were able to reduce the dose of corticosteroids they were receiving, while in 44%, an improvement in the endoscopic and imaging pictures was noticed. Three patients underwent surgery and 4 patients developed infections which were treated efficiently with antibiotics.

Miyatani *et al*[18] used a combination of ustekinumab plus upadacitinib, an oral selective Janus kinase inhibitor in 10 patients with CD with refractory active disease accompanied or not by extraintestinal manifestations. Five of the 6 patients with active CD and 2 of the patients with extraintestinal manifestations experienced clinical remission. Side effects during the 6-month follow-up were minimal (mainly upper respiratory infections).

In a retrospective study of 32 CD and 18 UC patients who received CT with biologic or micromolecular agents, Glassner *et al*[12] described that significantly more patients under CT were in clinical and endoscopic remission compared to baseline status. Erythrocyte sedimentation rate and C-reactive protein also showed significant value reduction. Side effects occurred in 26% mainly related to upper respiratory tract infections.

Interleukins 12 and 23 are known to play an important role in intestinal homeostasis and the pathogenesis of IBD. Their systematic study led to the development of monoclonal antibodies that target the p40 subgroup (ustekinumab and briakinumab) or p19 (risankizumab, guselkumab, brazikumab and mirikizumab). Feagan *et al*[19] investigated the possibility that the combined administration of guselkumab plus golimumab could be superior to monotherapy with either guselkumab or golimumab alone in patients with moderate to severe UC. Patients were randomized to receive guselkumab plus golimumab CT (72 patients), guselkumab alone (72 patients), or golimumab alone (71 patients). At the end of week 12, 83% of the combined treatment subjects achieved clinical remission compared to 61% and 75% of the other two groups, respectively. The most common side effects were upper respiratory infections, fever, anemia, and neutropenia. It therefore appears that CT with guselkumab plus golimumab is superior to monotherapy with guselkumab alone or golimumab alone.

Based on the results of the studies published so far, it appears that the combination of vedolizumab plus ustekinumab and vedolizumab plus anti-TNF-α factors are the preferred combinations in CD patients because they achieve satisfactory clinical results with an acceptable rate of side effects. The corresponding combinations for patients with UC concern the administration of vedolizumab plus anti-TNF-α factor or vedolizumab plus tofacitinib.

Despite the small number of patients included in the studies mentioned above, it appears that the combination of biological agents with a different mechanism of action is safe and effective in the treatment of patients with refractory IBD or patients with IBD and extraintestinal manifestations. It is clearly emphasized that it is necessary to carry out multicenter studies in a large number of patients as well as studies in rats using experimental models of colitis to investigate the possible effectiveness of the combination treatment, as well as the optimal dosage and duration of the administration of treatment[58].

Combination Treatment (combination of one biologic agent with one immunosuppressive drug): The combination of a biologic anti-TNF-α agent (mainly IFX and to a lesser extent adalimumab) with azathioprine appears to be more effective in CD patients than monotherapy with IFX or azathioprine[24,26,29,34]. The combination of vedolizumab with calcineurin inhibitors appeared also to be particularly effective in achieving remission in patients with active IBD[36]. Finally, the combination of vedolizumab with corticosteroids was shown to be more effective in inducing remission compared to vedolizumab or corticosteroids alone[37].

The side effects observed in the above studies are largely acceptable compared to the clinical benefit offered. Furthermore, it has long been known that the combination of IFX plus azathioprine, effectively prevents the formation of

antibodies against the biological agent. Clinicians should not avoid the combined use of these drugs when indicated.

Combination of first-line drugs (step-up therapeutic strategy): The use of antibiotics in severe UC flares remains a point of contention among experts, the majority of whom, at least theoretically, recommend avoiding their use in severe UC unless there is clear evidence of a septic condition. However, three recently published studies revisit the issue of combination antibiotic administration in UC flares[38-40].

In the case of patients with CD, the administration of antibiotics is easier, especially in patients with perianal disease.

In our opinion, in cases of patients with severe UC exacerbation, the possibility of Campylobacter *jejuni* infection should be carefully investigated by the gastroenterologists since this infection may worsen the clinical picture and delay remission of the disease.

The issue of antibiotic administration especially in patients with UC should be investigated in the future with multicenter, well-designed studies, in a large number of patients.

FACING THE FUTURE

As mentioned above, studies related to DBT are constantly being published, which combine biologics with small molecule agents (tofacitinib). Future studies should evaluate different dosages and combinations of drugs, different ways of administration, and different duration of treatment, emphasizing the possibility of adverse effects. The length of time of DBT administration should also be investigated, whether it should be administered only to induce remission or should also be administered as maintenance therapy. If DBT is used as a maintenance treatment then for how long and at what dosage should be administered? The type of administration should be at the same as in the induction phase or at reduced doses?

Another important field of research should be the possible combination of new pharmaceutical products that will equip our pharmaceutical quiver and which work with different mechanisms of action. Such drugs may be anti-IL-23 agents such as mirikizumab, risankizumab (Skyrizi, AbbVie), brazikumab and guselkumab, newer anti-integrin drugs such as etrolizumab and ontamalimab, as well as phosphodiesterase-4 inhibitors and sphingosine-1-phosphate receptor agonists. Currently, these drugs have good efficacy when administered individually, but it remains unknown whether they will work better in combination with older biological agents.

CONCLUSION

The need for the administration of combination biologic agents is constantly being established. After all, in daily clinical practice, regardless of whether there is not a sufficient number of randomized clinical studies investigating the effect of the simultaneous administration of established pharmaceutical agents (*e.g.*, corticoids, mesalazine, antibiotics, probiotics or immunosuppressants), this practice is widely applied. Patients with IBD require long-term and expensive treatment that should achieve important and difficult therapeutic goals such as the absence of symptoms, avoidance of complications and surgeries, prevention of disability and restoration of their quality of life.

Combination therapies appear to be effective in certain categories of patients, such as patients with refractory disease or patients with extraintestinal manifestations, although the treatment may be associated with an increased risk of adverse effects and malignancies.

The use of newer combinations, the application of new biomarkers and artificial intelligence, and clinical trials to establish efficacy during follow-up are necessary to implement with the aim of adopting new more effective therapeutic strategies in patients with resistant IBD.

The existing studies of combined use of biological agents lack the evidence of perfection that characterizes studies of single agents probably because there is no adequate financial support. Long-term safety data are also lacking. There is an urgent need in the near future for studies in sufficient numbers of patients with resistant disease and/or difficult-to-treat extraintestinal manifestations in which all possible combinations of biologic agents or biologic agents with other already established pharmaceutical agents, including immunosuppressants, are used.

At present the majority of studies suggest that no particularly serious adverse effects have been observed as a result of the use of DBT. On the other hand, it becomes apparent that with the explosive increase in the number of available biological agents, the possibilities of creating many and different combinations will become much easier in the future.

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EDITORIAL

Interplay between metabolic dysfunction-associated fatty liver disease and renal function: An intriguing pediatric perspective

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Abstract

Over recent years, the nomenclature of non-alcoholic fatty liver disease has undergone significant changes. Indeed, in 2020, an expert consensus panel proposed the term "Metabolic (dysfunction) associated fatty liver disease" (MAFLD) to underscore the close association of fatty liver with metabolic abnormalities, thereby highlighting the cardiometabolic risks (such as metabolic syndrome, type 2 diabetes, insulin resistance, and cardiovascular disease) faced by these patients since childhood. More recently, this term has been further replaced with metabolic associated steatotic liver disease. It is worth noting that emerging evidence not only supports a close and independent association of MAFLD with chronic kidney disease in adults but also indicates its interplay with metabolic impairments. However, comparable pediatric data remain limited. Given the progressive and chronic nature of both diseases and their prognostic cardiometabolic implications, this editorial aims to provide a pediatric perspective on the intriguing relationship between MAFLD and renal function in childhood.

Key Words: Metabolic (dysfunction) associated fatty liver disease; Renal; Function; Children; Obesity

Core Tip: Metabolic (dysfunction) associated fatty liver disease (MAFLD) has been closely linked to a wide spectrum of cardiometabolic consequences. Among these, accumulating data demonstrated an association between MAFLD and renal function in children with obesity. Worthy of note, a shared pathophysiology has been reported with a pivotal role of insulin-resistance in this dangerous interplay among obesity, renal hemodynamics, and metabolic derangements. Considering the relevant clinical and prognostic implications of this association, an increased awareness of this growing health concern for clinicians is needed.

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INTRODUCTION

In 2020, an expert panel proposed replacing the nomenclature of non-alcoholic fatty liver disease (NAFLD) with the more inclusive term metabolic (dysfunction) associated fatty liver disease (MAFLD) to underscore the pathogenic link between the disease and metabolic derangements in both adults and children[1,2]. According to the definition[2], the acronym MAFLD was introduced to strengthen the association of pediatric hepatic steatosis (diagnosed by biopsy with histological evaluation, imaging, or blood biomarkers) with at least one of the following criteria: (1) Excess adiposity; (2) Prediabetes or type 2 diabetes (T2D); and (3) Evidence of metabolic dysregulation[2].

Similar to NAFLD, the prevalence of MAFLD has been increasing worldwide in parallel with the obesity epidemic since childhood[1-3]. Current estimates suggest that approximately a quarter of the global adult population presents with MAFLD, while pediatric data indicate a global prevalence of 34% among children and adolescents with obesity in the general population, and 45% among peers in the obesity clinical setting[3]. Given this, MAFLD represents a major health and economic concern.

Indeed, MAFLD has been closely linked to cardiovascular disease (CVD) risk, but recent evidence also supports its role as a predictor of atherosclerosis, heart failure, T2D, and cancer-related mortality[1,3]. Consistent with previous evidence for NAFLD[4,5], a higher incidence of chronic kidney disease (CKD) has also emerged in patients with MAFLD[6-10], underscoring the impact of the disease on cardiometabolic health. Notably, robust adult data document a stronger association of MAFLD with CKD than NAFLD[10,11]. However, similar evidence linking MAFLD to CKD is still limited in childhood[12,13], though the underlying complex interplay among liver, kidney, adiposity, and metabolic dysfunction has been highlighted in these young patients[14-17]. Therefore, in this editorial, we aim to discuss the available evidence in the field to provide a perspective on the relevant clinical and prognostic implications of the interplay between MAFLD and CKD in children with obesity.

THE PATHOPHYSIOLOGICAL COMPLEXITY OF THE INTERPLAY BETWEEN MAFLD AND KIDNEY

Although the interplay of several determinants such as genetics, lifestyle, and environmental factors has been well-documented in the development of MAFLD, its exact pathogenesis remains to be fully elucidated[1-3]. In particular, certain features of MAFLD, such as systemic inflammation, metabolic dysfunction, and vascular dysfunction, have been found to act as mediators in the tangled inter-organ crosstalk underlying the close association of MAFLD with extrahepatic diseases (*e.g.*, CVDs, cognitive impairment, thyroid dysfunction, and CKD)[1,3,6,8]. On this basis, the relationship between MAFLD and renal function is unsurprising given their shared pathogenic factors such as low-grade inflammation, oxidative stress, and insulin resistance (IR)[8,9,18] (Figure 1).

In this complex pathophysiological puzzle, the contribution of genetics has also been well-documented[8,9,19]. The risk variant rs738409 C>G of the Patatin-like phospholipase domain-containing 3 gene represents a major genetic determinant of MAFLD in both adults and children[19-21]. Additionally, other polymorphisms such as the transmembrane 6 superfamily member 2 loss-of-function variant rs58542926[22] and the rs641738 C>T variant in the membrane-bound O-acyltransferase 7 gene[23,24] have been found to increase susceptibility to MAFLD[8,19]. Notably, recent evidence also supports a multifaceted role of these polymorphisms in renal health[21,22,24,25].

Furthermore, a prominent pathogenic role for inflammation needs to be underlined[26], as demonstrated by the inclusion of C-reactive protein among MAFLD diagnostic criteria[1,2]. Indeed, both chronic low-grade inflammation and oxidative stress lead to hepatic fibrosis by exacerbating pro-inflammatory signaling pathway activation[18,26], which in turn promotes endothelial dysfunction[18]. Consequently, this deleterious cycle impairs renal function by increasing glomerular permeability and proteinuria[18,27]. Notably, this increase in systemic inflammation might also affect cardiovascular health[6,8,18].

To complicate matters, IR - as a shared pathophysiological factor - plays a central role in the development of both MAFLD and CKD[28,29]. In fact, certain processes mediated by IR, such as an increased release of free fatty acids and an altered secretion of adipokines, affect both the liver and kidney[18,30-32].

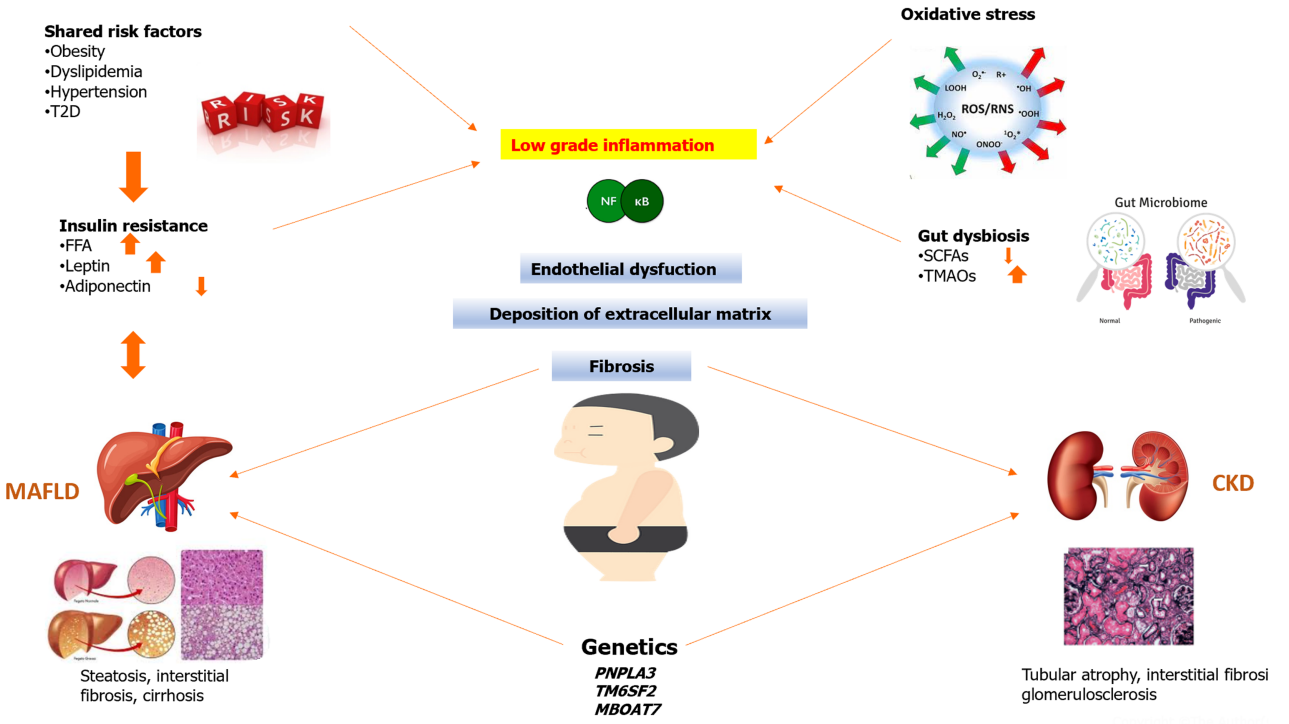

Figure 1 The complex pathophysiological interplay between metabolic (dysfunction) associated fatty liver disease and renal function. T2D: Type 2 diabetes; FFA: Free fatty acids; MAFLD: Metabolic (dysfunction) associated fatty liver disease; CKD: Chronic kidney disease; ROS: Reactive oxygen species; RNS: Reactive nitrogen species; SCFAs: Short chain fatty acids; TMAOs: Trimethylamine-N-oxides; PNPLA3: Patatin-like phospholipase domain-containing 3; TM6SF2: Transmembrane 6 superfamily member 2; MBOAT7: Membrane-bound O-acyltransferase 7.

Interestingly, recent insights into the complex pathogenic interplay between MAFLD and CKD highlight a role for the gut-liver-kidney axis, suggesting an intriguing contribution of gut microbiota to the development and progression of both diseases[18,33,34]. This realizes a vicious circle among dysbiosis, IR, inflammation, and oxidative stress[18,32]. In particular, metabolites derived from the gut microbiota such as trimethylamine-N-oxides[34,35] exert systemic effects impairing both liver[33,34] and renal function[35]. Additionally, the crosstalk between the liver and kidney also affects the production of short-chain fatty acids[36], further amplifying the underlying pathophysiological processes (*e.g.*, inflammation, IR, and oxidative stress) of MAFLD and CKD[36-38].

MAFLD AND CARDIOMETABOLIC HEALTH: THE RENAL PERSPECTIVE

MAFLD has been widely recognized as a strong predictor of all-cause mortality including CVD-related mortality[39-42]. More interestingly, recent data have unveiled an intriguing correlation between MAFLD and renal impairments[43-45], particularly CKD[18,38,40].

Several shared pathophysiological factors such as metabolic abnormalities (*e.g.*, obesity and IR), inflammation, adipokines, gut dysbiosis, and genetics contribute to liver and renal damage development[4,18,19,38]. In particular, IR appears to play a central role in the tangled interplay between MAFLD and CKD through various molecular pathways[6,8,18,38]. This exacerbates not only hypertension and atherogenic dyslipidemia but also activates renin-angiotensin system (RAS), which is closely related to endothelial dysfunction[6,8]. RAS activation further amplifies both proinflammatory and pro-coagulant states by releasing numerous mediators[6,8,18,38].

Moreover, evidence supports not only the intricate link between MAFLD and kidney function[8,43-45] but also the severity of hepatic damage with specific renal parameters in adults[46-48]. Indeed, recent studies have revealed associations between liver fibrosis, assessed *via* transient elastography, and kidney dysfunction parameters such as urinary albumin-to-creatinine ratio and estimated glomerular filtration rate (eGFR)[46-48]. However, research exploring the association between MAFLD and renal damage in pediatric populations remains limited[12,13].

Valentino *et al*[12] examined a cohort of Italian children with MAFLD and CKD during the initial coronavirus disease 2019 pandemic lockdown. After a six-month follow-up, children with MAFLD and CKD exhibited lower eGFR levels and an overall worse cardiometabolic profile compared to those without MAFLD[12]. De Groot *et al*[13] showed that children with a liver fat fraction > 2% and MAFLD presented with a worse cardiometabolic risk profile including higher blood pressure levels (as renal injury expression) compared to both children with a liver fat fraction < 2% and ≥ 2% liver fat without MAFLD. Of note, children with a liver fat fraction > 2% and MAFLD had an increased odds of clustering cardiometabolic risk factor compared to those with liver fat fraction < 2% independently of MAFLD presence (odds ratio = 7.65, 95% confidence interval: 5.04-11.62)[13]. In line with adult findings[42-44], these results underscore an association between MAFLD and renal damage in childhood[12,13]. Nevertheless, further large-scale studies are warranted to

validate this intriguing link comprehensively.

CONCLUSION

Recently, several lines of evidence have supported not only a pathogenic link between renal health and metabolic dysfunction but also its prognostic impact, both in children[12,13,18] and adults[18,40-43]. A complex inter-organ crosstalk sustained by metabolic impairments (*e.g.*, IR, visceral adiposity) has been supposed to be responsible for the relationship of MAFLD with extrahepatic diseases, including CKD[18]. More specifically, an intricate interplay among cardiometabolic risk factors, genetics, lipid nephrotoxicity, and hemodynamic changes has likely been implied in the relationship between MAFLD and renal impairment, but knowledge gaps in its exact pathophysiology still remain[8,18].

Compared to NAFLD, a better diagnostic and prognostic performance in identifying subjects at greater risk of hepatic and extra-hepatic complications has been demonstrated for the more inclusive MAFLD definition[8,18,41,48]. Based on these premises, children with MAFLD should receive more attention from clinicians through a multidisciplinary assessment that takes into account the intriguing MAFLD-associated multi-organ crosstalk.

Given the prognostic implications of the tangled relationship of MAFLD with extra-hepatic diseases[2,40-43], more scientific efforts are needed in this research area for a deeper understanding of the intricate interplay of molecular pathways contributing to hepatic and renal damage. On the other hand, this might also implement strategies for overall MAFLD management (including prevention, diagnosis, and treatment) to optimize patient outcomes since childhood.

FOOTNOTES

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EDITORIAL

Advancements in hemostatic strategies for managing upper gastrointestinal bleeding: A comprehensive review

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Abstract

Upper gastrointestinal (GI) hemorrhage presents a substantial clinical challenge. Initial management typically involves resuscitation and endoscopy within 24 h, although the benefit of very early endoscopy (< 12 h) for high-risk patients is debated. Treatment goals include stopping acute bleeding, preventing rebleeding, and using a multimodal approach encompassing endoscopic, pharmacological, angiographic, and surgical methods. Pharmacological agents such as vasopressin, prostaglandins, and proton pump inhibitors are effective, but the increase in antithrombotic use has increased GI bleeding morbidity. Endoscopic hemostasis, particularly for nonvariceal bleeding, employs techniques such as electrocoagulation and heater probes, with concerns over tissue injury from monopolar electrocoagulation. Novel methods such as Hemospray and Endoclot show promise in creating mechanical tamponades but have limitations. Currently, the first-line therapy includes thermal probes and hemoclips, with over-the-scope clips emerging for larger ulcer bleeding. The gold probe, combining bipolar electrocoagulation and injection, offers targeted coagulation but has faced device-related issues. Future advancements involve combining techniques and improving endoscopic imaging, with studies exploring combined approaches showing promise. Ongoing research is crucial for developing standardized and effective hemorrhage management strategies.

Key Words: Upper gastrointestinal bleeding; Hemostasis; Endoscopy; Probe; Spray; Clip

Core Tip: Endoscopic hemostasis for nonvariceal upper gastrointestinal bleeding primarily involves electrocoagulation and heater probes, though monopolar electrocoagulation raises tissue injury concerns. Newer methods such as Hemospray and Endoclot offer mechanical tamponade but with limitations. First-line treatments currently include thermal probes and hemoclips, with over-the-scope clips gaining traction for larger ulcers. The gold probe, merging bipolar electrocoagulation and injection, targets coagulation effectively but has device-related issues. Future progress lies in integrating techniques and enhancing endoscopic imaging. Research is vital to establish standardized, effective hemorrhage management strategies.

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INTRODUCTION

Upper gastrointestinal (GI) hemorrhage is a substantial clinical challenge that often necessitates urgent medical intervention. Notably, bleeding from ulcers halts spontaneously in at least 80% of cases without specific intervention[1]. However, the annual incidence of upper GI bleeding (UGIB) ranges from 0.05% to 1%, with several patients succumbing to this condition[2]. This underscores the critical need for effective hemostatic treatments and the importance of ongoing research in this area.

Initial management of GI hemorrhage typically involves resuscitation and subsequent decisions regarding therapeutic interventions. Current guidelines advocate endoscopy within 24 h for patients with UGIB. Whether high-risk patients would benefit more from very early endoscopy (within 12 h) remains open to debate[3,4]. Studies have indicated that restrictive fluid resuscitation (employing a delayed or smaller fluid volume) is not inferior to more aggressive fluid resuscitation strategies (involving early or larger fluid volumes) in terms of mortality[5].

The primary goals of treating GI hemorrhage are two-fold: Halting acute bleeding episodes and preventing rebleeding. Achieving these goals requires a multimodal approach that includes endoscopic, pharmacological, angiographic, and surgical therapies. Various pharmacological agents, such as vasopressin, secretin, prostaglandins, somatostatin, and proton pump inhibitors have been effectively employed for the management of GI hemorrhage[6]. However, studies have also highlighted that the increasing incidence of combined pharmacological agents such as antithrombotics has increased the morbidity from GI bleeding, indicating that direct intervention is often essential[1]. Angiographic therapies, including gel foam and vasopressin, have been used, although they can potentially lead to complications, such as ischemia, stenosis, infarction, perforation, and abscess formation[7].

Endoscopic hemostasis has become the accepted standard of care for individuals presenting with acute nonvariceal upper GI hemorrhage. Techniques such as monopolar electrocoagulation, bipolar electrocoagulation, and heater probes have also been used. Although effective, monopolar electrocoagulation can cause a greater degree of tissue injury than bipolar electrocoagulation, which has been a source of concern[8]. This technique uses a single electrical circuit to heat and stop the bleeding, which can sometimes harm surrounding tissues. However, bipolar electrocoagulation, which uses two electrical points to create a more focused and less damaging heat, and heater probes, are particularly useful for arterial bleeding of < 2 mm, which addresses the requirements of the majority of patients with ulcer bleeding[9].

Injection strategies vary depending on the agent used, with mechanisms of action that may include vasoconstriction, tamponade effects, induction of platelet aggregation, sclerosis, thrombosis, and/or tissue desiccation[10]. At our center, during endoscopic submucosal dissection (ESD) and peroral endoscopic myotomy procedures, we initially apply injection techniques when the bleeding source is unclear, primarily to induce vasoconstriction and tamponade effects, thereby reducing bleeding before proceeding to precise clipping. An innovative approach involves the use of a powder, specifically Hemospray (HS, TC-325; Cook Medical, Bloomington, IN, United States). When this powder comes into contact with blood, it absorbs water and works together to create a mechanical barrier by acting cohesively and adhesively to form a mechanical tamponade. This process helps to stop bleeding effectively. By absorbing fluid, HS enhances clot formation by deforming and packing erythrocytes, concentrating activated platelets with clotting factors, and interacting with the fibrin matrix[11]. However, its residence time is limited to 24 h or less, and it does not induce tissue healing. Consequently, TC-325 monotherapy might not be adequate for treating ulcers with high-risk stigmata but can be useful as a temporary measure to halt bleeding. In such cases, a second-look endoscopy or an additional hemostatic technique is recommended[12]. Our team primarily uses TC-325 particularly after procedures such as ESD, when there is substantial bleeding, or when the depth of post-procedural ulcers suggests a risk of delayed bleeding.

Endoclot (EC; Micro-Tech Europe, Düsseldorf, Germany) is made of starch-derived compounds composed of absorbable hemostatic polysaccharides. Similar to HS, upon contact with blood, EC initiates a dehydration process that leads to a concentration of clotting factors, platelets, and erythrocytes, thereby accelerating the physiological clotting cascade and the formation of a mechanical shell of the gelled matrix that adheres to the bleeding tissue[13].

Currently, the first-line therapy for ulcer-related GI bleeding includes the use of thermal probes and through-the-scope clips with or without the adjunctive use of submucosal epinephrine injection[14]. However, there are limitations to achieving hemostasis during active hemorrhage, especially in cases of large and/or cratered fibrotic ulcers in anatomically challenging locations. Recently, over-the-scope clips (OTSCs) have emerged as a promising alternative. These

larger-caliber clips, composed of nitinol, a metal known for its shape memory effect and high-grade elasticity, allow high-pressure closure of larger mucosal areas. OTSCs capture deeper tissue layers and may enhance hemostasis[15]. Given meta-analysis findings that OTSCs reduce 30-d rebleeding in UGIB, their use has increased[16]. Our endoscopy team primarily employs OTSCs in cases of bleeding where perforation is suspected.

The gold probe (Microvasive, Boston, MA, United States) represents a considerable advancement in this field of research. This probe combines bipolar electrocoagulation with an internal injection mechanism, which makes it particularly useful for targeting specific coagulation sites. When positioned perpendicular to the mucosa, the probe at 20 W and 40 W for less than 6 s caused coagulation confined to the mucosal layer, whereas at 9 s, submucosal coagulation occurred, and at 80 W for more than 15 s, coagulation extended to the muscular layer[17]. The design of the gold probe aims to reduce kinking, thus facilitating its advancement and providing better en face and tangential tamponades. The integration of injection and thermal hemostasis into a single catheter is intended to reduce catheter exchange and the procedural time. The precise spacing of the electrode pairs helps to control the coagulation depth, and the rounded distal tip is designed to facilitate effective coagulation at various tip positions. However, issues have arisen with gold probes, particularly when used with or without injection, including energy delivery, followed by material separation, fracture of the probe tip, arcing, missing components, bending of the tips, and device detachment.

Given these frequent device-related problems, the use of a gold probe, even without adverse effects on patients, might not be advisable. In endoscopic hemostasis therapy, two major complications, i.e., uncontrollable bleeding and viscus perforation, are rare; however, their potential occurrence must be considered in every case.

The future direction for advancement in hemostatic techniques involves evaluating the combination of different methods for their safety and effectiveness. One study explored a conventional combined technique involving saline adrenaline injection followed by heater probe application. This method involved injection of saline adrenaline, followed by application of a heater probe to the ulcer at the site of the visible vessel. Subsequent energy pulses up to 30 J were delivered until the vessel was completely flattened or ablated. This approach was superior to TC-325 monotherapy. One potential strategy involves the application of TC-325 multiple times over the first few days following a conventional combined technique. Furthermore, the combination of OTSCs with various hemostatic tools merits further investigation to determine their efficacy. Advancements in endoscopic imaging techniques are crucial for more accurate and effective bleeding control. For example, the recent introduction of the Olympus X1500 endoscope model and its use of rapid diagnostic imaging (RDI) is a step quicker identification of bleeding sites. However, there is no complete consensus on the diagnosis and management of hemorrhages, highlighting the need for ongoing research to develop standardized and quantified indications and methods.

CONCLUSION

In conclusion, endoscopic hemostatic techniques, much like the once-prominent but now less used gold probe, are evolving. While methods such as injection and clipping have been consistently employed in the past, there is a growing scope for newer techniques such as OTSCs and hemostatic powder for managing UGIB. The introduction of the Olympus X1500 with its RDI adds another dimension to diagnosing and managing bleeding foci. Continuous research is necessary to further explore and optimize the application of these hemostatic techniques.

FOOTNOTES

Author contributions: Lee YA contributed to conceptualization, writing–original draft, formal analysis, investigation, and editing; Cho JY contributed to conceptualization, supervision, writing–review, and editing; All authors approved the final version of the article.

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EDITORIAL

Understanding autoimmune pancreatitis: Clinical features, management challenges, and association with malignancies

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Abstract

In this editorial we comment on the article by Jaber *et al*. Autoimmune pancreatitis (AIP) represents a distinct form of pancreatitis, categorized into AIP-1 and AIP-2, characterized by obstructive jaundice, lymphoplasmacytic infiltrate, and fibrosis. AIP-1, associated with elevated immunoglobulin G4 (IgG4) levels, exhibits higher relapse rates, affecting older males, while AIP-2 is less common and linked to inflammatory bowel disease. AIP is considered a manifestation of IgG4-related systemic disease, sharing characteristic histological findings. Steroids are the primary treatment, with emerging biomarkers like interferon alpha and interleukin-33. AIP poses an increased risk of various malignancies, and the association with pancreatic cancer is debated. Surgery is reserved for severe cases, necessitating careful evaluation due to diagnostic challenges. AIP patients may have concurrent PanINs but display favorable long-term outcomes compared to pancreatic cancer patients. Thorough diagnostic assessment, including biopsy and steroid response, is crucial for informed surgical decisions in AIP.

Key Words: Autoimmune pancreatitis; Immunoglobulin G4-related disease; Pancreatic cancer; Surgery

Core Tip: Autoimmune pancreatitis (AIP) is a unique form of pancreatitis, categorized into AIP-1 and AIP-2, with distinct clinical characteristics and associations. AIP-1, linked to elevated immunoglobulin G4 (IgG4) levels, exhibits higher relapse rates and predominantly affects older males, while AIP-2 is less common and associated with inflammatory bowel disease. Recognized as a manifestation of IgG4-related systemic disease, AIP poses an increased risk of malignancies, especially gastric, colorectal, and bladder cancers. Despite ongoing debates about the association with pancreatic cancer, careful diagnostic evaluation, including biopsy and response to steroids, is crucial for informed decision-making regarding surgery. AIP patients may have concurrent PanINs but generally experience better long-term outcomes compared to pancreatic cancer patients. Steroids remain the primary treatment, and emerging biomarkers like interferon alpha and interleukin-33 offer promising avenues for monitoring and managing AIP.

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INTRODUCTION

A unique kind of pancreatitis known as autoimmune pancreatitis (AIP) is typified by fibrosis and lymphoplasmacytic infiltration, obstructive jaundice, and a noticeable reaction to steroids. AIP can be classified into two types, AIP-1 and AIP-2. AIP-1 is associated with elevated levels of immunoglobulin G4 (IgG4) and it is prevalent in the seventh decade. It has a greater male-to-female ratio, particular histological findings, a higher relapse rate, and a propensity to manifest as painless jaundice. The prevalence of type 1 AIP in Japan has increased significantly, with an incidence of 3.1 per 100000 people, a sex ratio of 2.94 (males to females), and a mean age at diagnosis of 64.8 years[1-3]. On the other hand, AIP-2 is less common, it is more prevalent in the 4th-5th decade and is characterized by specific histological findings. It is often presented with acute pancreatitis, obstructive jaundice, and is less associated with IgG4 but more with inflammatory bowel disease (IBD).

Yoshida first proposed the idea of AIP in 1995. Clinical manifestations of the condition frequently include obstructive jaundice, emaciation, exhaustion, and stomach discomfort. Serum IgG4 levels are raised, and imaging examinations show irregular stricture of the pancreatic duct and widespread or segmental enlargement of the pancreas[2]. According to histological study, AIP is typified by lymphocytic sclerosing pancreatitis, which is characterised by a substantial infiltration of IgG4-positive plasma cells and CD4-positive T cells around the pancreatic duct, resulting in occlusive fibrosis and stenosis[4].

IgG4-related systemic disease (IgG4-RD) is a fibroinflammatory disease involving multiple organs. IgG4-RD is a chronic, inflammatory disease with increased serum IgG4 concentrations and fibrosis as well as enlarged organs infiltrated with IgG4+ plasmacytes. Type 1 AIP is now considered a pancreatic manifestation of systemic IgG4-RD, with characteristic histological findings including lymphocyte and IgG4-positive plasma cell infiltration, fibrosis, and obliterative phlebitis[3,5,6]. Approximately 60% to 80% of AIP patients demonstrate obstructive jaundice with sclerosing cholangitis, resembling primary sclerosing cholangitis, pancreatic cancer, or cholangiocarcinoma.

AIP 1 has a difficult to predict recurrence risk. However, some factors, including diffuse pancreas enlargement, persistently high IgG4 levels, slow IgG4 level decrease, increased IgG4 level following glucocorticoid treatment, and proximal IgG4-associated sclerosing cholangitis, may be suggestive of recurrence[1,4]. According to recent research, interleukin-33 (IL-33) and interferon alpha (IFN-α) may function as biomarkers for IgG4-RD and AIP. The first-line recommended treatment for AIP is steroids, which over 90% of individuals with type 1 AIP have remission from[2,4].

The pathogenesis of type 1 AIP has yet to be deciphered. Toll-like receptors (TLRs) play a role in the development of AIP-1, with overexpression observed in the pancreas and salivary glands of AIP and IgG4-RD patients 3. TLR-7 upregulation is documented in IgG4-RD, and plasmacytoid dendritic cells (pDCs) may contribute to the pathogenesis of AIP/IgG4-RD. Unregulated production of IFN-α by pDCs is associated with AIP. Apoptosis inhibitor of macrophages may serve as a potential biomarker for autoimmune and inflammatory diseases with tissue fibrosis, including IgG4-RD and AIP[3,5].

Pancreatic parenchymal and ductal imaging, serology, pancreatic histology, involvement of other organs, and response to steroid therapy are among the diagnostic criteria for AIP[7]. Serum IgG4 concentration, though not ideal, is a commonly used biomarker, and other serological markers, such as hypocomplementemia, antinuclear antibodies, rheumatoid factor positivity, and specific autoantibodies, have been investigated. The cutoff value for serum IgG4 is confirmed at 140 mg/dL[7]. Histopathological evaluation involves assessing IgG4 immunostaining of the duodenal papilla, neutrophil infiltration and molecular markers like IFN-α and IL-33, which are considered valuable for diagnosis and monitoring. Radiological imaging reveals diffuse pancreatic swelling, irregular narrowing of the main pancreatic duct, and characteristic patterns on dynamic computed tomography and contrast-enhanced magnetic resonance imaging. Endoscopic retrograde cholangiopancreatography typically shows narrowing of the main pancreatic duct, and magnetic resonance cholangiopancreatography may display multiple intermittent absences[7]. Endoscopic ultrasound (EUS) fine-needle aspiration is a useful technique for tissue sampling, especially in cases of focal AIP or when serum IgG4 levels are within normal limits[7].

AFTEREFFECTS OF IGG4 RELATED PANCREATITIS

AIP is correlated with an increased incidence of various diseases. Haghbin *et al*[2], over a follow-up period ranging from 6 months to 22 years observed in their systematic review and metanalysis, that among 2746 AIP patients, 9.6% were found to have malignancies. Notably, 3.7% of these malignancies were diagnosed before or concurrently with AIP, while 4.6% were diagnosed after AIP. This emphasizes the importance of early and comprehensive cancer surveillance in AIP patients, as malignancies can manifest at any stage. Huggett *et al*[8] discovered that 13 of 115 patients with type 1 AIP (11.3%) had a malignancy, indicating a statistically significant risk of malignancy in individuals with type 1 AIP. However, the incidence of pancreatic cancer was low[2,8]. The most prevalent cancers in the AIP population were stomach, colorectal, and bladder cancer. These findings indicate the possibility that AIP is linked to malignancies outside of the affected organ[2,8].

The potential association between pancreatic cancer and AIP is a subject of ongoing debate. While AIP is a rare form of chronic pancreatitis, concerns have been raised, particularly in type 1 AIP, characterized by a higher incidence of disease relapse after steroid therapy. Existing studies, present conflicting evidence on the link between AIP and pancreatic cancer. Hart *et al*[9] observed an incidence of pancreatic cancer in type 1 AIP Patients of 0.51% with 5 diagnoses out of 978 type 1 AIP patients. Xiang *et al*[10] report an incidence of pancreatic cancer up to 6.7% and Macinga *et al*[11], in their review, observed that out of the 33 cases of patients with pancreatic cancer and AIP, 67% of those had their diagnosis at median period of 66.5 months (2-186 m), explaining the need for more systematic research in this area and a long term cancer surveillance[10,11].

Investigating the link between AIP-2 and IBD, particularly the increased rates of CRC in IBD, revealed no significant difference in CRC prevalence between the two types of AIP. This suggests a need for further exploration of the relationship between AIP-2, IBD, and CRC[2]. Non-malignant manifestations of AIP included sclerosing cholangitis, sialadenitis, and retroperitoneal fibrosis. Sclerosing cholangitis was observed up to 80% of AIP patients, emphasizing the diverse systemic impact of AIP[4]. About 60% to 80% of AIP patients presented with obstructive jaundice, often associated with sclerosing cholangitis. Additionally, AIP has been linked to IgG4-associated sclerosing cholangitis, resembling primary sclerosing cholangitis, pancreatic cancer, or cholangiocarcinoma in cholangiography features. It is known that various cancers have been reported in association with AIP or IgG4-RD, suggesting a complex interplay. However, there is a lack of prospective studies supporting the notion that AIP or IgG4-RD may develop as paraneoplastic syndromes.

Long-term chronic inflammation, a hallmark of AIP, is recognized for its role in carcinogenesis. Contrary to expectations, some studies showed no occurrence of pancreatic cancer in AIP and/or IgG4-RD patients during follow-up periods. The risk of malignancies in AIP patients appears higher within the first year after diagnosis, and treating coexisting cancers has been associated with preventing AIP relapse. Miyagawa *et al*[5] suggest that in some cases AIP as well as IgG4 related disease may be a paraneoplastic syndrome for other cancers, activating an IGg4 immune response.

ROLE OF SURGERY IN AIP

Typically, surgery is not the primary option for AIP. Nevertheless, it should be contemplated when suspicions of malignant or premalignant lesions persist following a comprehensive diagnostic evaluation. The International Consensus Diagnostic Criteria (ICDC) advocates nonoperative approaches for managing AIP patients, with surgical intervention reserved for those experiencing severe symptoms, or to resolve associated biliary strictures[12].

Ensuring a thorough evaluation before surgery is crucial to optimize patient selection and avoid unnecessary procedures. A heightened clinical suspicion for AIP plays a crucial role in refining the decision-making process for surgical interventions. Early consideration of the potential for AIP is vital to prevent unnecessary surgeries or diagnostic procedures[12]. In cases where AIP is strongly suspected, it is recommended to measure serum IgG4 levels and perform a biopsy, with EUS-guided trucut biopsy being the preferred modality. When the biopsy fails to provide a clear diagnosis or raises suspicions of malignancy, a brief 2-wk steroid treatment may serve as an alternative to surgery. A swift resolution of imaging abnormalities, typically within two weeks, is observed in cases of AIP. In contrast, if a malignant lesion is present, no change in operability is anticipated during this brief period[13]. If the diagnosis of AIP is confirmed, and malignancy is ruled out, opting for rituximab treatment may prove to be a more effective alternative, resolving biliary manifestations without the need for surgery[12]. Moreover, in cases where AIP is strongly suspected, it is advisable to undergo a biopsy. If the biopsy results do not indicate features suggestive of malignancy, a brief course of steroid treatment should be contemplated. Corticosteroid therapy is the primary treatment for AIP, achieving remission in over 90% of cases. In cases involving a solid mass suggestive of malignancy, there is a consensus that obtaining biopsy proof is not obligatory before proceeding with resection[14]. However, patients with borderline resectable disease should confirm malignancy before undergoing neoadjuvant therapy and exploration for resection. The International Study Group of Pancreatic Surgery recommends surgical resection of a pancreatic solid mass without the need for histopathological confirmation of malignancy[15].

Despite a comprehensive preoperative assessment, a subset of patients persists in whom malignancy cannot be ruled out without resorting to pancreatic resection. Complicating the decision to operate or not, it is crucial to recognize that AIP is a rare disease[14]. In contrast, pancreatic ductal adenocarcinoma (PDAC) ranks as the 11th most common cancer globally, accounting for 4.5% of all cancer-related deaths in 2018, while the preoperative diagnosis of AIP does not eliminate the possibility of simultaneous pancreatic cancer[12]. Retrospective analysis revealed that approximately 23% of patients had concurrent malignant or premalignant lesions with AIP[14]. National Comprehensive Cancer Network

guidelines suggest that when a high suspicion for malignancy exists, surgical intervention is considered in pancreatic resections performed for suspected malignancy and the final histological examination often reveals benign conditions in 8% to 10% of patients. AIP constitutes approximately one-third of these cases, making up 2.5% of all pancreatic resections [16].

These occurrences need to be prevented since life-threatening complications might exacerbate the postoperative course after pancreatic resections. One of the most serious complications is a pancreatic fistula, which can have septic and hemorrhagic effects[12]. Strategies such as modifying anastomotic techniques, placing pancreatic duct stents, and prophylactic use of somatostatin analogs have been employed to enhance postoperative outcomes. Despite a notable decrease in perioperative mortality with increased surgical experience and improved critical care management, morbidity rates remain elevated, particularly in high-volume centers[17]. Despite the growing understanding of AIP, the number of patients undergoing pancreatic resection and being incorrectly diagnosed with pancreatic malignancy hasn't decreased over time, as observed[14].

Ikeura *et al*[18] highlighted the challenges in diagnosing AIP. The diagnostic yield of the ICDC without histology and response to steroids was low in focal AIP, diagnosing AIP in only 20% of patients suspected of having cancer. However, a steroid trial after excluding pancreatic cancer boosted ICDC's diagnostic yield to 73%, even in the lack of histology. This highlights the significance of pancreatic core needle biopsy or surgical excision in the remaining individuals[18]. In a study involving 114 European patients with surgically treated AIP, the relapse rate was 41.2% for AIP type 1 and 15.4% for AIP type 2[19]. In AIP, concurrent PanINs are frequently identified, with approximately 25% of patients in this study having incidental PanINs[16]. This aligns with findings from other studies indicating a notable occurrence of PanINs in individuals undergoing surgery for benign pancreatic conditions. These precursor lesions likely elevate the risk of future PDAC development, suggesting that a subset of patients may benefit from surgical resection. In comparison to patients undergoing resection for confirmed malignancy, those with AIP exhibit remarkable long-term outcomes[16]. In this cohort, only 18% experienced serious sequelae (≥ Clavien-Dindo grade 3), compared to 30% in a recent multi-institutional study of PDAC. Furthermore, whereas the 5-year survival rate for pancreatic cancer is normally approximately 8%, virtually all AIP patients survive at the 5-year milestone, with a rate around 100%[16].

CONCLUSION

In conclusion, AIP is intricately linked to malignancies, emphasizing the importance of vigilant cancer surveillance in AIP patients. The association with various cancers beyond the pancreas suggests a systemic impact, and further research is needed to elucidate the complex relationship between AIP, IgG4-RD, and cancer. Surgery is reserved for severe cases or unresolved biliary strictures, with careful consideration due to the challenges in distinguishing AIP from malignancy. While AIP patients may have concurrent PanINs, they exhibit better long-term outcomes and survival compared to pancreatic cancer patients. Thorough diagnostic evaluation, including biopsy and response to steroids, is crucial in refining the decision-making process for surgery in AIP.

FOOTNOTES

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EDITORIAL

Probiotics: Shaping the gut immunological responses

Eirini Filidou, Leonidas Kandilogiannakis, Anne Shrewsbury, George Kolios, Katerina Kotzampassi

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Abstract

Probiotics are live microorganisms exerting beneficial effects on the host's health when administered in adequate amounts. Among the most popular and adequately studied probiotics are bacteria from the families *Lactobacillaceae*, *Bifidobacteriaceae* and yeasts. Most of them have been shown, both *in vitro* and *in vivo* studies of intestinal inflammation models, to provide favorable results by means of improving the gut microbiota composition, promoting the wound healing process and shaping the immunological responses. Chronic intestinal conditions, such as inflammatory bowel diseases (IBD), are characterized by an imbalance in microbiota composition, with decreased diversity, and by relapsing and persisting inflammation, which may lead to mucosal damage. Although the results of the clinical studies investigating the effect of probiotics on patients with IBD are still controversial, it is without doubt that these microorganisms and their metabolites, now named postbiotics, have a positive influence on both the host's microbiota and the immune system, and ultimately alter the topical tissue microenvironment. This influence is achieved through three axes: (1) By displacement of potential pathogens *via* competitive exclusion; (2) by offering protection to the host through the secretion of various defensive mediators; and (3) by supplying the host with essential nutrients. We will analyze and discuss almost all the *in vitro* and *in vivo* studies of the past 2 years dealing with the possible favorable effects of certain probiotic genus on gut immunological responses, highlighting which species are the most beneficial against intestinal inflammation.

Key Words: Probiotics; Lactobacillaceae; Bifidobacteriaceae; Saccharomyces; Intestinal inflammation; Immune responses

Core Tip: Probiotics, such as *Lactiplantibacillus plantarum* and *Saccharomyces cerevisiae*, exert remarkable anti-inflammatory properties on the gut's immune responses. These beneficial microorganisms not only restore immunity markers but also enrich the gut's microbiota, crucial for a healthy microbial balance. Incorporating probiotics or foods rich in these beneficial microorganisms, particularly in conditions such as inflammatory bowel disease, holds promise for restoring gut health, boosting the immune system, and alleviating inflammation.

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INTRODUCTION

According to the current definition, "probiotics are live microorganisms that, when administered in adequate amounts, confer a health effect on the host"[1,2]. They can be found either as pure forms supplied by various pharmaceutical companies or as essential parts of everyday foods, mainly fermented, such as cheese, yogurt, beer and others[3]. Some of the most well-studied probiotics are bacteria, such as the *Lactiplantibacillus plantarum*, and yeasts, such as *Saccharomyces*, for which extensive research has shown that they possess anti-inflammatory and wound healing properties[4,5].

Probiotics are considered to exert their beneficial effects not only on the host's cells, but also on its natural microbiota composition. Since the onset of the Microbiome Project, several species of bacteria and yeasts have been identified, which has led to the extensive identification/characterization, of the human microbiota composition, found on the cutaneous and mucus surfaces of the human body[6]. Microbiota has been proved to be essential for the host's survival, not only acting as a defense mechanism against potential pathogenic microorganisms, but also providing viable nutritional supplementation[7]. Over time, it has been shown that the microbiota population is 10 times greater than the total number of cells composing the human body, and, as a result, it has been proposed that humans are "symbiotic" organisms, living in harmony with their microbiota[8].

One of the mucosal surfaces with the best characterized and most well-studied microbiota is that of the intestine. Under healthy conditions, the composition of the intestinal microbiota is balanced and beneficial to the host, but in pathological situations, this balance can be disturbed, leading to dysbiosis, with potentially harmful consequences to the host[9]. Such a dysbiosis has been confirmed in patients suffering from inflammatory bowel disease (IBD)[9]. IBDs – Crohn's Disease (CD) and Ulcerative Colitis (UC) – are characterized by chronic relapsing inflammation of the gastrointestinal tract, with various immunological, genetic and environmental factors involved in its pathogenesis[9]. One such environmental factor is the intestinal microbiota. Patients with IBDs have been shown to have a distinct, altered microbiota composition, lower in diversity compared to healthy individuals, and, in many cases, attempts to restore their microbiota composition to a healthy state have proved beneficial for the patient's health. Such attempts have been through either fecal microbial transplantation, where fecal matter from healthy donors is transplanted into patients with IBD to restore their microbiota to a healthy state, or through probiotic supplementation[10].

One of the major beneficial effects of probiotics is their ability to modulate the immunological responses of the host. This can be accomplished by: (1) Displacement of potential pathogens *via* competitive exclusion; (2) offering protection to the host through the secretion of various defensive mediators; and (3) supplying the host with essential nutrients[7]. In this Editorial, we will analyze and discuss almost all the *in vitro* and *in vivo* studies published on the past 2 years which have investigated the possible favorable effects of certain genus of probiotics on gut immunological responses (Figure 1), in an effort to highlight which are the most beneficial in relation to intestinal inflammation.

THE GENUS LACTIPLANTIBACILLUS

Lactiplantibacillus (formerly known as *Lactobacillus*), is a genus of Gram-positive bacteria that has been associated with favorable outcomes for the host. Many of its members have been proved to be essential players in the food industry[11]. One of the most well-known is *Lactiplantibacillus plantarum* (*L. plantarum*), which has been found to thrive in a wide range of environments, including fermented foods, several types of meat and plants, as well the mammalian gastro-intestinal tract[12]. Regarding its effects on the gastro-intestinal tract, *L. plantarum* has been shown to boost wound healing and promote anti-inflammatory processes[13].

In a series of studies, any researchers have highlighted the immune-related properties of *L. plantarum* in the gastro-intestinal tract. Our research group has shown that *L. plantarum* may participate in the alertness of the intestinal immune system, as it seems to mildly upregulate specific chemokines in subepithelial myofibroblasts[14]. In a cyclophosphamide-induced immunosuppressive animal model, Zeng *et al*[15] observed that the administration of *L. plantarum* led to the enhancement of the immune system through the restoration of inflammatory cytokines and immune markers in the spleen[15]. In another animal model of antibiotic-induced diarrhea, Liang *et al*[16] showed that the administration of *L. plantarum* ELF051 significantly improved the animals' health, by downregulating pro-inflammatory signaling pathways and cytokines, such as interleukin (IL)-1β, upregulating the anti-inflammatory ones, such as IL-10, and by enriching the

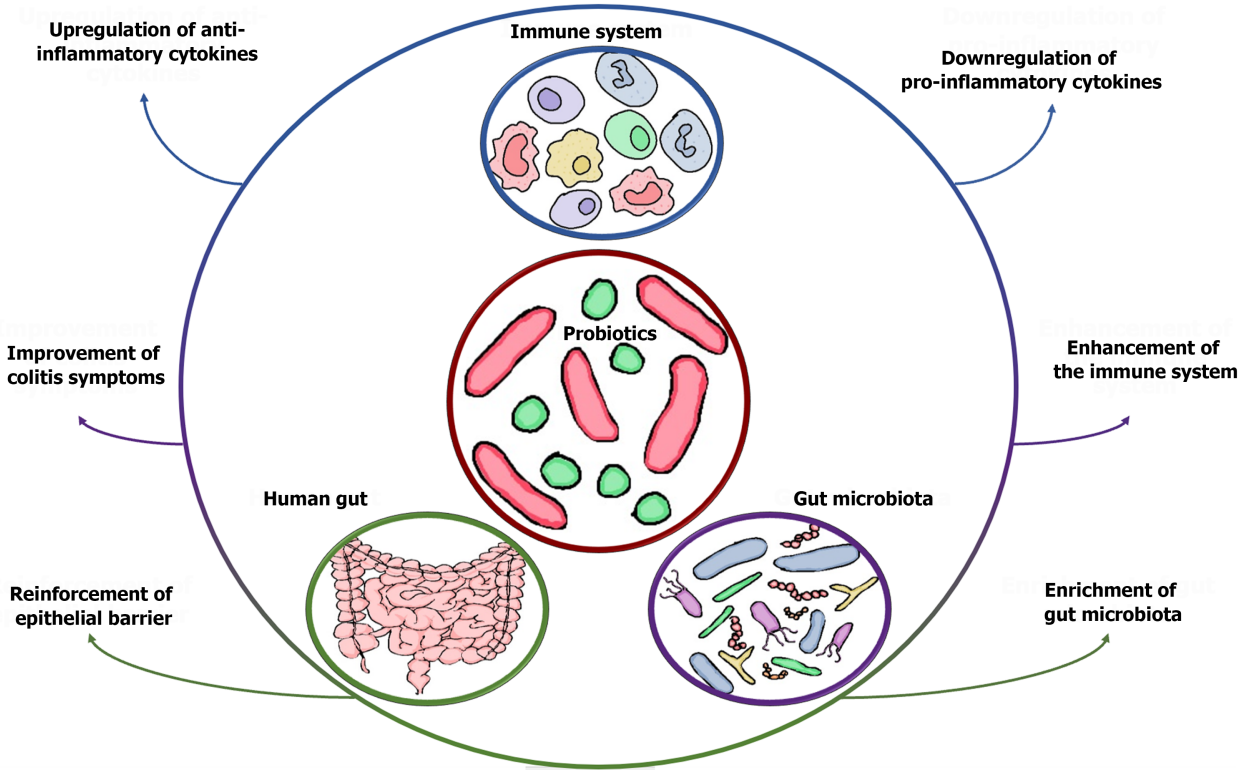

Figure 1 The beneficial effects of probiotics on the immune system, intestinal microbiota and the human gut. Probiotics promote the upregulation of anti-inflammatory cytokines, induce the downregulation of pro-inflammatory ones, improve the colitis symptoms, enhance the immune system, reinforce the epithelial barrier and favor the enrichment of the gut microbiota.

diversity of the topical gut microbiota[16]. In the same way, *L. plantarum* YRL45, a bacteriocin-producing probiotic, has been reported to favorably regulate the immune system of mice by elevating the immunoglobulins sIgA, IgA and IgG levels and by upregulating the expression of epithelial markers mucin 2, zonula occludens-1 and junctional adhesion molecule 1[17].

Similar results come from a study investigating the immunoregulatory effects of *L. plantarum* CRL681 and CRL1506 in enterotoxigenic *Escherichia coli* (*E. coli*) infection in mice; both strains found to favorably modulate the intestinal innate immune response and increase resistance to *E. coli*, ultimately leading to reduced counts of *E. coli* in the gastrointestinal tract[18]. In another study, Li *et al*[19] showed that the administration of *L. plantarum* in *E. coli*-infected mice led to a significant improvement in disease status, since it significantly stopped weight loss and restored the flattened mucosa in the jejunum, findings probably related to the significant downregulation of the proinflammatory cytokines[19]. This improvement was further enhanced when *L. plantarum* was administered along with two other probiotics, *Bifidobacterium longum* and *Pediococcus acidilactici*[19], supporting the idea that a combined rather than a single regime of probiotics is more effective.

Heat-killed fractions and proteins from *L. plantarum* 299v, now named postbiotics[20], have also proved to have anti-inflammatory properties, in the same way as live bacteria: In an lipopolysaccharides (LPS) *in vitro* model they were found to downregulate the pro-inflammatory cytokine IL-18[21], thus exerting immunomodulatory properties on the immune responses. The anti-inflammatory properties of *L. plantarum* are also highlighted by the proteomic study of Cufaro *et al* [22]: *L. plantarum* C904 was once again found able to downregulate *in vitro,* to a considerable degree pro-inflammatory cytokines, including IL-2, IL-5, IL-6, and interferon (IFN)-γ, in inflamed intestinal epithelial cells[22].

Another subspecies, the panda-derived *L. plantarum* BSG201683, when added to LPS-treated intestinal epithelial cell cultures, has been shown to strengthen their integrity, downregulate pro-inflammatory and upregulate anti-inflammatory cytokines, such as IL-10[23]. The undoubted anti-inflammatory effects of *L. plantarum* are further supported by the study of Ren *et al*[24] Mice with either acute or chronic dextran sulfate sodium (DSS)-induced colitis, when given *L. plantarum,* presented with overall improved health; the colitis symptoms improved, as did both the oxidative stress and inflammatory response[24]. The results from an LPS-induced colitis model in mice are similar; *L. plantarum* was able to ameliorate colitis, not only by counteracting its symptoms, but also by downregulating pro-inflammatory cytokines and by strengthening the epithelial barrier integrity[25].

THE GENUS LACTOBACILLUS

Lactobacillus acidophilus (*L. acidophilus*) is the most well-known probiotic species from the genus *Lactobacillus*, also having

significant anti-inflammatory properties. We have previously shown that *L. plantarum* and *L. acidophilus* are involved in the immunological alertness of the intestinal tissue, as it slightly upregulated specific chemokines in subepithelial myofibroblasts[14]. One of the metabolites of *L. acidophilus*, the indole-3-lactic acid, has been shown to act beneficially during DSS-colitis in cesarean-born mice, as its administration led to decreased intestinal inflammation and increased type-3 innate lymphoid cells (ILC3) and IL-22 Levels[26]. When bone marrow dendritic cells were co-cultured with *L. acidophilus*, it was found that the probiotic promoted both the production of IL-17 by $CD4^+$ T cells, and IL-22 by ILC3 cells [27].

In another study, *L. acidophilus* was found to improve DSS-induced colitis when in tandem with another probiotic strain, *Veillonella ratti*. By working together, these probiotics significantly restored lost body weight and colon length in mice, perhaps through short chain fatty acids (SCFA) production, while also improving overall disease activity, by downregulating pro-inflammatory cytokines and oxidative stress markers and upregulating anti-inflammatory factors [28]. The protective effects of *L. acidophilus* have also been emphasized by Aximujiang *et al*[29]: When *L. acidophilus* was given in combination with the Chinese medicine Huan Kui Le suspension, the protective effect against colitis was dramatically enhanced. The immune responses shifted towards immunoregulatory ones, and were supported by the upregulation of IL-13 and transforming growth factor-β and the downregulation of IFN-γ, the microbiota composition being once again enriched with beneficial bacteria[29].

THE GENUS LACTICASEIBACILLUS

Lacticaseibacillus rhamnosus, (formerly *Lactobacillus rhamnosus*, *L. rhamnosus*), has also been shown to exert anti-inflammatory and immunoregulating properties. Indeed, postbiotic (*i.e.* heat-killed fractions and proteins from the bacteria) of *L. rhamnosus* were also reported to downregulate the pro-inflammatory cytokine IL-18 in the LPS *in vitro* model of Magryś *et al*[21], previously described, but also to upregulate the anti-inflammatory cytokine IL-10, which *L. plantarum* failed to do[21].

Chemotherapy to fight cancer is known to induce gastrointestinal tract inflammation[30,31]. When *L. rhamnosus* was given as pretreatment, it was shown to mitigate the inflammatory responses; Nenu *et al*[32] showed that the combinational therapy of regorafenib and *L. rhamnosus* for the treatment of hepatocellular carcinoma in mice resulted in a significant reduction of inflammation and gut permeability[32], while Alsholi *et al*[33] reported that the administration of *L. rhamnosus* alleviated cisplatin-induced mucositis in an animal model[33]. Lu *et al*[34] also reported that *L. rhamnosus* GG postbiotic and antiprogrammed cell death 1 (anti-PD1) immunotherapy had better results in colorectal cancer treatment in relation to anti-PD1 immunotherapy alone. The authors observed increased populations of MHC II^+ DC cells, $CD4^+$ and $CD8^+$ T cells in the tumor sites[34], suggesting that the probiotic extracellular vesicles could boost the immune system in favor of the host, to fight the cancerous cells.

Apart from its effects on cancer-induced inflammation, *L. rhamnosus* has been also shown to have beneficial anti-inflammatory properties in DSS-colitis; Kim *et al*[35] found that the administration of *L. rhamnosus* KBL2290 in mice could ameliorate colitis by restoring body weight and colon length, reducing disease activity, and downregulating pro-inflammatory cytokines, while at the same time upregulating the anti-inflammatory IL-10[35]. The results from an LPS-induced inflammation in Caco-2 cell culture are similar; *L. rhamnosus* was found to counteract the detrimental effects of LPS on Caco-2 cells, thus enhancing their survival, reducing the inducible oxidative stress, inducing the expression of tight junction proteins and by downregulating pro-inflammatory cytokines[36]. Tomotsune *et al*[37] supported the option that the beneficial immunomodulatory effects of *L. rhamnosus* are not necessarily promoted through its adhesion to the epithelial barrier, but can also be exerted without binding to the mucus, possibly through its secreted products[37].

Finally, the beneficial effects of *L. rhamnosus* are not limited to the immune system. Chen *et al*[38] showed that this probiotic may favorably influence the epithelial barrier during sepsis, thus increasing survival time; which may occur through the increase of intestinal stem cells proliferation[38].

THE GENUS LIGILACTOBACILLUS

Ligilactobacillus [formerly known as the *Lactobacillus salivarius* (*L. salivarius*) group], are lactic acid producing, Gram-positive bacteria, commonly found in fermented foods[39]. *L. salivarius* has been characterized as a potential probiotic, due to its anti-inflammatory properties. Carbonne *et al*[40] showed that *L. salivarius* CNCM I-4866 was able to downregulate a number of pro-inflammatory markers both *in vitro* and *in vivo*, strengthen the epithelial barrier and inhibit the adherence of various intestinal pathogens to the host's epithelial cells[40].

THE GENUS LIMOSILACTOBACILLUS

The genus *Limosilactobacillus*, (formerly known as *Lactobacillus*) comprises several species able to possibly exert favorable outcomes for the host[41]. The most well-studied *Limosilactobacillus* species is *Lactobacillus fermentum* (*L. fermentum*).

Two species of the *Limosilactobacillus* genus, *L. fermentum* MN410703 and MN410702, were used to investigate whether they effectively inhibit enteric pathogens to prevent environmental enteropathy. The authors concluded that both strains have strong anti-inflammatory properties, and thus prevent chronic gut inflammation through over-expression of IL-6

and IL-10 *in vitro* and downregulation of the pro-inflammatory cytokine IL-8. Additionally, both strains were found to exert strong antagonistic properties on pathogens, adhesion to HT-29 cells, and inhibition of pathogen adherence to HT-29 cells[42].

L. fermentum has also been shown to protect against chemotherapy-induced gut permeability by regulating the expression and localization of tight-junction proteins and LPS-induced inflammation and by downregulating various pro-inflammatory cytokines. A number of authors have suggested that *L. fermentum* treatment could alleviate the adverse effects of chemotherapy in patients with colon cancer[43]. In the case of DSS-colitis, *L. fermentum* has also shown promising results as it improved the overall health of mice by restoring their lost body weight and colon length, but also by strengthening the epithelial barrier integrity through the expression of the tight-junction proteins. Additionally, it shifted the immune responses by promoting the T regulatory and suppressing the T inflammatory cells[44]. In a similar animal model of colitis, *L. fermentum* was shown to counteract inflammation by targeting the nuclear factor kappa B (NF-κB) signaling pathway, and thus downregulating several pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF)-α and IL-1β[45].

In another study which compared the possible anti-inflammatory properties of *Limosilactobacillus mucosae* (*L. mucosae*) and *Lactobacillus amylovorus* with another lactic acid bacterium, the *L. mucosae,* it was found to predominate; although both probiotics downregulated several pro-inflammatory cytokines in mice with DSS-colitis, *L. mucosae* had better effects in alleviating the colitis symptoms[46].

THE GENUS LEVILACTOBACILLUS

Levilactobacillus (formerly also known as *Lactobacillus*) is a genus of Gram-positive bacteria found mainly in fermented foods and in the composition of the intestinal microbiota[47]. One of its species, *Levilactobacillus brevis*, has been found to have promising anti-inflammatory properties, as Kim *et al*[48] showed that, when given, in inflamed HT-29 intestinal epithelial cells it resulted in the reduction of IL-8 and NF-κB levels, possibly through targeting the extracellular signal-regulated kinase and Akt signaling pathways[48].

THE GENUS BIFIDOBACTERIUM

Bifidobacterium, a genus of Gram-positive bacteria, is found both in food and in the gastrointestinal tract. Several of its species are also considered as beneficial probiotic supplements[49]. In an experimental model of excisional cutaneous trauma in rats, we have previously shown that *Bifidobacterium longum* (*B. longum*) can promote wound healing and especially angiogenesis[13]. However, its gut mucosal effect on inflammation is still under discussion. Li *et al*[19] found that the administration of *B. longum* in *E. coli*-infected mice successfully restored the lost body weight and colon length; however, it only downregulated the pro-inflammatory cytokines IL-6 and TNF-α, but not IL-1β[19]. In a similar study, where the animals were infected with *Plasmodium berghei*, *B. longum* was able to counteract the infection by diminishing parasitemia, reducing the levels of the pro-inflammatory cytokines IFN-γ and TNF-α in the serum and by enhancing an anti-inflammatory profile in the animals[50].

On the other hand, *B. longum* BAA2573 was observed to ameliorate DSS-induced colitis in mice, restoring the body weight and colon length by means of decreasing a number of disease activity markers, such as neutrophil infiltration, while, at the same time, also improving the gut microbiota diversity[51]. This action pathway leads us to believe that *B. longum* may be beneficial only in certain types of inflammation, or that it may exert its anti-inflammatory properties only when combined with other probiotics. In the study of Yue *et al*[52], when *B. longum* was given with *Bifidobacterium bifidum* to mice with LPS-induced colitis, the beneficial result was even greater than when either one was given alone. Working together these two probiotics managed to boost the immune system by elevating the IgA levels in the serum, along with increasing populations of $CD4^+/CD8^+$ T and dendritic cells. They also succeeded in strengthening the epithelial barrier by means of promoting the expression of mucus and tight junction proteins and, additionally, downregulated various pro-inflammatory cytokines[52].

Finally, bioengineered probiotics have been reported to have even greater beneficial effects on the host than simple bacteria strains[53]. One such example is *B. longum* fortified with artificial enzymes, which can lead to much reduced intestinal inflammation and enriched microbiota diversity[54].

Bifidobacterium bifidum (*B. bifidum*), on its own, has also been shown to ameliorate DSS- and trinitrobenzene sulfonic acid-induced colitis in mice. In both studies, *B. bifidum* was found to improve the overall health of the mice by restoring their body weight and colon length, strengthening the epithelial barrier integrity and the immune profile, downregulating pro-inflammatory factors and upregulating the anti-inflammatory ones[55,56]. In the case of DSS-colitis, the favorable effects of *B. bifidum* were speculated to arise through the activation of the aryl hydrocarbon receptor in the intestine[56].

Bifidobacterium breve (*B. breve*) has also been reported to possess anti-inflammatory properties. In particular, Park *et al* [57] showed that the administration of *B. breve* in two different animal models of colitis (DSS and dinitrobenzene sulfonic acid) had favorable effects, leading to the amelioration of disease severity. It increased the number of goblet cells in the intestinal epithelium and also strengthened the epithelial barrier by upregulating the mRNA of tight-junction proteins [57]. In an animal model of ileitis, the administration of a four probiotic formula comprising *Saccharomyces boulardii*, *L. rhamnosus*, *Lactobacillus acidophilus*, *B. breve* plus amylase led to a significant improvement in overall health: Disease activity was reduced, gut microbiota was enriched, and the immune system significantly boosted[58]. Nonetheless, these

beneficial effects were not observed when amylase was not given[58]. It is proposed that amylase might play a significant role in inhibiting the biofilm formation by the potentially harmful bacteria.

Bifidobacterium lactis (*B. lactis*) is known to exert promising probiotic properties. Our research group has highlighted that this probiotic may contribute to the immunological alertness of the intestinal tissue through the upregulation of specific chemokines in subepithelial myofibroblasts[14]. In the study by Lan *et al*[59], the administration of *B. lactis* in mice with DSS-induced colitis led to the amelioration of the disease (weight loss and disease activity scores were reduced), but more significantly, several pro-inflammatory cytokines were downregulated[59], strongly highlighting the anti-inflammatory properties of *B. lactis*.

Bifidobacterium pseudocatenulatum (*B. pseudocatenulatum*) is a less studies species. Wang *et al*[60] reported that the administration of *B. pseudocatenulatum* in mice with DSS-induced colitis led to an overall amelioration of the disease, the integrity of the epithelial barrier was found to be strengthened through the upregulation of tight-junction proteins and mucus production, oxidative stress was decreased by the promotion of the expression of several antioxidant enzymes and inflammation was decreased through the downregulation of pro-inflammatory cytokines and the upregulation of the anti-inflammatory IL-10[60].

Finally, *Bifidobacterium animalis* (*B. animalis*) subsp. *lactis* BLa80 has been investigated for its possible therapeutic properties in IBD. *B. animalis* subsp. *lactis* BLa80 was shown to have a favorable effect in DSS-induced colitis in mice, as it not only reduced the histological disease scores and restored the colon length, but it also downregulated pro-inflammatory cytokines and enriched the microbiota composition[61].

THE GENUS PEDIOCOCCUS

Pedioccocus is a Gram-positive, lactic acid bacterium which plays a significant role in the food fermentation process[62]. Li *et al*[19] found that the administration of *Pediococcus acidilactici* in *E. coli*-infected mice successfully restored body weight and colon length, as well as downregulating the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α[19].

Another species, *Pediococcus pentosaceus* (*P. pentosaceus*) CECT8330, was investigated for its possible anti-inflammatory properties in a mouse model of DSS colitis[63,64]. The administration of *P. pentosaceus* resulted in the restoration of lost body weight and colon length, and in the reduction of disease activity and inflammation[63]. However, it is of exceptional interest to mention that *P. pentosaceus* is involved in an early switch in macrophage phenotype from the pro-inflammatory M1 to M2, in parallel with the downregulation of IL-1β levels. This finding is evidence of the acceleration of the inflammatory phase during the healing process[64].

THE GENUS AKKERMANSIA

Akkermansia is an aerotolerant anaerobe, Gram-negative bacteria, capable of growing on a viscus substrate such as mucin [65]. Although it was first isolated in 2004, it is known that it has the ability to degrade intestinal mucin glycoproteins, a process leading to the production of SCFA. Additionally, it promotes mucin turnover, thus strengthening the mucosal barrier and reducing gut permeability[66]. Liu *et al*[67] highlighted the beneficial properties of *Akkermansia muciniphila* (*A. muciniphila*) in a mouse model of antibiotic-induced diarrhea. *A. muciniphila* was found to reduce diarrhea incidents, to enhance the epithelial barrier integrity and to enrich the microbiome and metabolome profiles. Regarding the inflammation status, it resulted in the upregulation of anti-inflammatory markers, GPR109A and SLC5A8, and the downregulation of pro-inflammatory TNFα, IFN-γ, IL1β, and IL6[67]. The favorable anti-inflammatory effects of *A. muciniphila* have also been underlined by Daniel *et al*[68], being found to ameliorate the emulsifier-induced inflammation in mice through the reduction of inflammatory cell infiltration and histology scores[68].

Finally, another study underlines the favorable immunomodulatory effects of *A. muciniphila* on the gut-brain axis. A culture medium of Caco-2 intestinal epithelial cells, when pre-treated with *A. muciniphila*, exhibited an inhibitory effect on pro-inflammatory cytokine production in human microglial cells[69].

THE GENUS ROUXIELLA

The genus *Rouxiella* was first described back in 2015 by Le Flèche-Matéos *et al*[70]. One of its members, the *R. badensis* subsp. *acadiensis* (Canan SV-53), is thought to exert immunomodulatory effects, as Shahbazi *et al*[71] showed that the administration of *R. badensis* subsp. *acadiensis* in healthy mice resulted in an increase in serum IgA, a decrease in several pro-inflammatory cytokines and an increase in the anti-inflammatory subset population of cells in the small intestine[71], all probably contributing to immune system boosting. In the next experiment by the same research group on LPS-induced colitis in mice, the administration of *R. badensis* subsp. *acadiensis* led to the amelioration of disease severity through anti-inflammatory effects that mainly targeted the epigenetic mechanisms of several genes involved in the differentiation of Th17 cells[72].

THE GENUS ROSEBURIA

Roseburia are Gram-positive anaerobic bacteria. *R. intestinalis* exerts immunomodulatory effects and is a major butyrate producer in the gut[73]. Kang *et al*[74] showed that *R. intestinalis* may favor cytotoxic $CD8^+$ T cell populations during colorectal cancer, and thus boost the immune system towards fighting tumorous cells[74].

THE GENUS TETRAGENOCOCCUS

The genus *Tetragenococcus* comprises Gram-positive facultative anaerobic lactic acid bacteria[75], and among its members, *Tetragenococcus halophilus* (*T. halophilus*) has been shown to potentially protect against intestinal inflammation. In a DSS-induced colitis animal model, the administration of *T. halophilus* led to an overall amelioration of disease activity, but more significantly, it also reduced the activation of several immune-related cell populations, leading to decreased production of the pro-inflammatory cytokine IL-1β[76].

THE GENUS FAECALIBACTERIUM

The genus *Faecalibacterium* is an extremely oxygen-sensitive commensal butyrate-producer, populating the most anaerobic parts of the GI tract of mammals and is generally recognized as a biomarker of intestinal health[77]. *Faecalibacterium prausnitzii* (*F. prausnitzii*) has been found to be decreased in patients with IBD[78], and thus its (further) supplementation may lead to favorable outcomes. Indeed, in an animal model of DSS-induced colitis, the administration of Treg cells exposed to *F. prausnitzii* resulted in the amelioration of colitis, with decreased disease scores and significantly reduced inflammation[79].

THE GENUS ENTEROCOCCUS

Enterococcus is a genus of lactic acid bacteria, the species of which exert both harmful and beneficial effects[80], thus some species may be considered as possible probiotic supplements. Zheng *et al*[81] showed that *Enterococcus faecium* (*E. faecium*) could have a protective effect against the enterotoxigenic *Escherichia coli* infection, since it enhances the expression of tight-junction proteins and downregulates pro-inflammatory cytokines[81]. Benvenuti *et al*[82] suggested that the administration of *E. faecium* in obese mice resulted in reduced inflammation and improvements in the integrity of the epithelial barrier[82]. Although these results seem promising this genus has not yet received the status of "Generally Recognized As Safe" (GRAS) due to a number of potential health risks[80].

THE GENUS CLOSTRIDIUM

Clostridium is a genus of Gram-positive anaerobic bacteria and, similar to the *Enterococcus* genus, it includes both harmful and beneficial species[83]. *Clostridium butyricum* (*C. butyricum*), one of its species, exerts possible anti-inflammatory effects in experimental colitis. Indeed, extracellular vesicles (postbiotic) from this species have been shown to have anti-inflammatory properties both *in vitro* and *in vivo*, by suppressing the pro-inflammatory signaling pathways of mitogen-activated protein kinase and NF-κB through the restoration of the expression of miR-199a-3p[84]. The reduction in inflammation by means of suppression of the myeloid differentiation primary response 88 and NF-κB signaling pathways upon administration of *C. butyricum*, was also observed in an animal model of colorectal cancer[85], suggesting that *C. butyricum* could indeed be considered as a possible anti-inflammatory probiotic supplement.

THE GENUS SACCHAROMYCES

Saccharomyces is a yeast and *Saccharomyces cerevisiae* (*S. cerevisiae*) is one of the most well-known species, widely used in the food industry – brewer's yeast - but additionally serves as a very potent probiotic[86]. Regarding its role in regulating inflammation, Kil *et al*[87] showed that the administration of *S. cerevisiae* to mice with DSS-induced colitis resulted in the downregulation of neutrophil infiltration and the pro-inflammatory cytokine, TNF-α, and in upregulation of the expression of tight-junction proteins and the anti-inflammatory cytokine IL-10[87]. In transgenic rats subjected to ileocecal resection for Crohn's disease, when postoperative recurrence occurred, the administration of *S. cerevisiae* resulted in a reduction in the macroscopic and histological lesions in the anastomosis area, a decrease in *E. coli* LF82 adherence, as well as in downregulation of the pro-inflammatory IL-23 and IL-17 cytokines and upregulation of the anti-inflammatory IL-10 [88].

When four different yeasts were tested both *in vitro* and *in vivo*, *S. cerevisiae* predominate as shown to have the strongest anti-inflammatory properties. *S. cerevisiae* has also been shown to protect against experimental colitis, as the overall health of animals improved and several inflammation markers were downregulated[89].

Another yeast of the same genus is the *Saccharomyces boulardii*. We have also previously documented in relation to subepithelial myofibroblasts that it is implicated in the immunological alertness of the intestinal tissue, through a mild upregulation of specific chemokines[14].

CONCLUSION

Our assessment of the most studied probiotics led us to conclude that all genera and their species, in different ways, downregulate intestinal inflammation and enhance immune response, as follows (Table 1): Almost all genera downregulate pro-inflammatory cytokines production; less genera, mainly of *Lactobacillaceae* family upregulate the anti-inflammatory cytokines. *L. plantarum*, *L. acidophilus* and *L. rhamnosus*, *B. breve* and *B. lactis*, *R. badensis* ssp.acadiensis and *R.intestinalis* as well as *S. boulardii* enhance immune function through different pathways. Almost all genera strengthen the epithelial barrier integrity by restoring the expression of tight junction proteins and/or by promoting of mucus expression. *L. plantarum* and *L. salivarius*, *B. longum*, *B. breve*, and *B. animalis* ssp. *lactis* as well as *A. muciniphila* enhance the disturbed - due to disease - intestinal microbial diversity. *L. rhamnosus* and *B. pseudocatenulatum* promote the expression of antioxidant enzymes. *L. rhamnosus* promotes the proliferation and differentiation of intestinal stem cells; *B. breve* relieves intestinal inflammation through augmenting goblet cell regeneration; and *P. pentosaceus* shifts macrophage polarization toward the anti-inflammatory M2 phenotype. *Muciniphila* seems to exert neuroprotective effects through the 'gut-brain' axis. Almost all genera - through different mechanisms and pathways - alleviate experimental colitis symptoms.

Table 1 Probiotics on intestinal inflammation and immune responses

Probiotics	Immune system↑	Inflammatory cytokines		Epithelial barrier↑	Colitis symptoms ↓	Micro-biota diversity↑	Oxidative stress↓	Stem cells ↑	Goblet cells↑	M2 macrophages ↑	Gut-brain axis↑	Ref.
		↓Pro-	↑Anti-									
L. plantarum	+	+	+	+	+	+						[14-19, 21-25]
L. acidophilus	+	+	+		+							[14, 26-29]
L. rhamnosus	+	+	+	+	+		+	+				[21, 32-36, 38]
L. salivarius		+		+		+						[46]
L. fermentum		+	+	+	+							[42-45]
L. brevis		+										[48]
B. longum		+	+	+	+	+						[19, 50-52, 54]
B. bifidum		+	+	+	+							[55, 56]
B. breve	+			+	+	+			+			[57, 58]
B. lactis	+	+			+							[14, 59]
B. pseudo-catenulatum		+	+	+	+		+					[60]
B. animalis ssp. lactis		+			+	+						[61]
P. acidilactici		+			+							[19]
P. pentosaceus		+			+					+		[63, 64]
A. muciniphila		+	+	+		+					+	[67-69]

R. badensis ssp.acadiensis	+	+	+		+		[71, 72]
R. intestinalis	+						[74]
T. halophilus		+			+		[76]
F. prausnitzii		+			+		[79]
E. faecium		+	+	+			[81, 82]
C. butyricum		+					[84, 85]
S. cerevisiae		+	+	+	+		[87-89]
S. boulardii	+						[14]

The up↑ and down↓ arrows mean 'increase' and 'decrease', respectively.

FOOTNOTES

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MINIREVIEWS

Hepatocellular carcinoma and musculoskeletal system: A narrative literature review

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Abstract

Musculoskeletal alterations in hepatocellular carcinoma (HCC) are less common than liver-related complications. However, they can significantly impact the quality of life and overall prognosis of patients with HCC. The main obstacle in the clinical assessment of HCC-induced musculoskeletal alterations is related to effective and timely diagnosis because these complications are often asymptomatic and unapparent during routine clinical evaluations. This narrative literature review aimed to provide a comprehensive overview of the contemporary literature related to the changes in the musculoskeletal system in patients with HCC, focusing on its clinical implications and underlying etiopathogenetic mechanisms. Osteolytic bone metastases are the most common skeletal alterations associated with HCC, which could be associated with an increased risk of low-trauma bone fracture. Moreover, previous studies reported that osteopenia, sarcopenia, and myosteatosis are associated with poor clinical outcomes in patients with HCC. Even though low bone mineral density and sarcopenia are consistently reported as reliable predictors of pretransplantation and post-transplantation mortality in HCC patients, these complications are frequently overlooked in the clinical management of patients with HCC. Taken together, contemporary literature suggests that a multidisciplinary approach is essential for early recognition and clinical management of HCC-associated musculoskeletal alterations to improve patient prognosis. Further research into the mechanisms and treatment options for musculoskeletal complications is warranted to enhance our understanding and clinical management of this aspect of HCC.

Key Words: Hepatocellular carcinoma; Osteopenia; Osteoporosis; Sarcopenia; Bone metastases; Bone fragility

Core Tip: Musculoskeletal alterations have a powerful detrimental effect on the quality of life and prognosis of patients with hepatocellular carcinoma (HCC). The causes of HCC-induced musculoskeletal decline are complex and not yet fully understood. The biggest challenge in diagnosing HCC-related musculoskeletal changes is timely and effective diagnosis, as these alterations are often asymptomatic and may not be obvious during routine clinical evaluations. Therefore, a multidisciplinary approach to the clinical management of musculoskeletal alterations is essential in patients with HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, comprising 90% of patients with liver malignancy[1]. This type of malignancy typically develops due to end-stage chronic liver disease (around 80% of cases develop from cirrhosis)[2]. HCC rarely develops in the absence of liver cirrhosis or advanced liver fibrosis[3]. The highest incidence of HCC is found in Southeast Asia and North Africa where hepatitis B infection is endemic. In Western countries alcohol-associated and metabolic dysfunction-associated fatty liver disease and steatohepatitis are the predominant factors for HCC development[2,4-6].

Due to the aggressive nature of HCC, the prognosis of patients with HCC is poor (overall 5-year survival rate < 12%)[2], and HCC is the third leading cause of cancer-related deaths worldwide[1]. The estimated incidence of newly diagnosed patients with HCC is around 500000-1000000 per year, causing a global loss of 600000 lives each year[7]. Therefore, early identification of significant risk factors is essential to alter the disease course and improve patient survival and prognosis.

Screening programs for early detection of HCC improved survival in individuals with chronic liver disease who developed HCC[8]. Several prognostic systems have been developed over the years to identify the risk factors that could reveal poor prognosis in patients with HCC including[9]: The Barcelona Clinic Liver Cancer system[10]; the Cancer of the Liver Italian Program[11]; the Chinese University Prognostic Index score[12]; and the Child-Pugh score[10]. Still, all these scoring systems lack parameters considering the nutritional, functional, and performance status of patients with HCC[5]. Although long-term prognosis is dependent on the liver reserve and cancer staging[13], poor performance can significantly affect clinical outcomes in HCC patients. Therefore, the Eastern Cooperative Oncology Group scale was developed to provide an assessment of the performance and functional statuses in patients with HCC[14]. Recent advances in artificial intelligence-based risk calculations have enabled the integration of new risk factors that could significantly alter the clinical management of individuals with HCC[15].

The liver plays a central role in human metabolism, and patients with HCC are at a high risk of developing various complications. While the primary focus in the study of HCC traditionally revolves around liver-related complications (*e.g.*, hepatic encephalopathy, portal vein thrombosis, ascites, variceal bleeding, and obstructive jaundice), recent investigations have shed light on HCC-induced complications in other organs and systems, including the musculoskeletal system. HCC is almost three times more likely to appear in males than in females, with an incidence of 5.5/100000 in males and 2.0/100000 in females[2]. Thus, it is reasonable to predict that sex-specific distribution of musculoskeletal alterations (which are more common in postmenopausal females) could be shifted toward males with HCC.

In addition, it is known that the age distribution of patients with HCC is related to dominant viral hepatitis in the underlying population and the age at which it was acquired. However, it is important to note that HCC reaches its highest prevalence among individuals older than 65 years[2,7,16]. Considering that older age is a major risk factor for developing major musculoskeletal alterations (osteopenia, sarcopenia, and/or osteosarcopenia) and based on the global rise of an aging population[17-19], musculoskeletal complications are steadily becoming a major health concern in individuals with HCC.

Recognizing the importance of musculoskeletal health within the global health agenda, the Global Alliance for Musculoskeletal Health recently made substantial attempts to create a global roadmap for improving musculoskeletal health[20]. The first step on this journey is understanding the complexity of determinants that can affect musculoskeletal health and evaluating the particular health-burden contribution of each of these determinants in individuals who are healthy, aging, or with chronic diseases (Figure 1). Given that musculoskeletal complications are preventable, it is essential to fully understand the interconnections between HCC and musculoskeletal health, which can be beneficial for developing more effective and cost-efficient management strategies and increasing the quality of life for individuals with HCC (Figure 1).

This article aimed to provide a comprehensive narrative overview of the contemporary literature related to the changes in the musculoskeletal system in patients with HCC by focusing on its clinical implications and underlying etiopathogenetic mechanisms. Also, this review aimed to identify potential gaps in the current literature and suggest directions for future studies in HCC-associated musculoskeletal alterations.

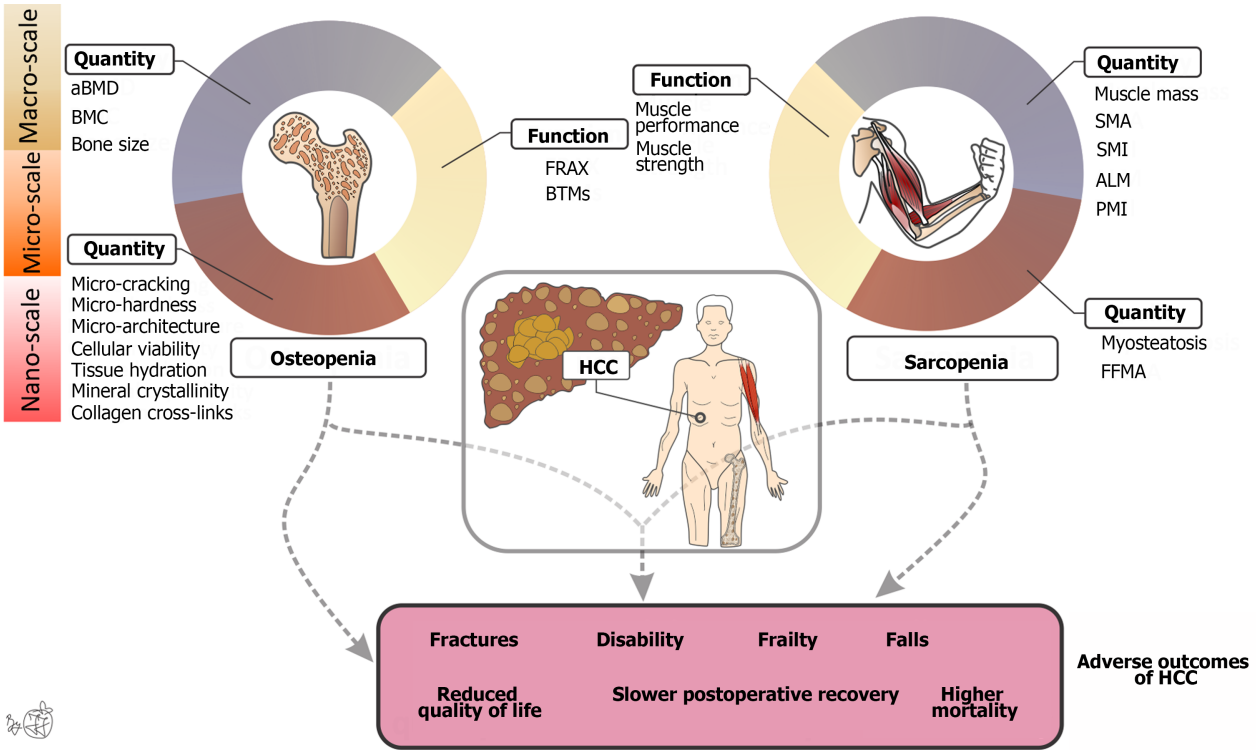

Figure 1 Role of musculoskeletal alterations in clinical management of patients with hepatocellular carcinoma. Various factors contribute to musculoskeletal decline in patients with hepatocellular carcinoma. A multiscale and multidisciplinary approach should be used to assess musculoskeletal health. aBMD: Areal bone mineral density; BMC: Bone mineral content; HCC: Hepatocellular carcinoma; FRAX: Fracture risk assessment tool; BTMs: Bone turnover markers; ALM: Appendicular lean mass; FFMA: Fat-free muscle area; PMI: Psoas muscle index; SMA: Skeletal muscle area; SMI: Skeletal muscle index.

LITERATURE SEARCH STRATEGY

An electronic search was performed using the PubMed/Medline, Embase, Cochrane, Web of Science, and CINAHL databases on November 25, 2023. To identify published articles on skeletal alterations in patients with HCC, we used the following search terms: "carcinoma, hepatocellular" OR "cancer, hepatocellular" AND "osteopenia" OR "osteoporosis" OR "bone mineral density" OR "bone metastases" OR "bone fracture". To identify published articles on muscular alterations in patients with HCC, we used the following search terms: "carcinoma, hepatocellular" OR "cancer, hepatocellular" AND "sarcopenia" OR "myosteatosis". Both authors independently reviewed the search results they obtained. Preclinical (basic science) and clinical studies written in English were included in this review. In cases of discrepancies, the dilemma was resolved through discussion, and both authors agreed with the final pool of studies included in the review.

SKELETAL-RELATED EVENTS IN PATIENTS WITH HCC

Bone metastases[21-24], pathological bone fractures[24,25], reduced bone mineral density (BMD)[26,27], hypercalcemia [28], and spinal cord compression[29] are among the most clinically relevant HCC-associated skeletal-related events.

The risk of bone metastasis in patients with HCC is not as prominent as in other common malignancies, such as gastric cancer, lung cancer, or breast cancer. There is a varying incidence of bone metastasis in patients with HCC of 3%-20%[30-32]. Substantial technological progress has been made in diagnosing and treating patients with HCC, which improved the overall survival rate, and bone metastases have become more commonly observed in recent years. Bone metastasis is reported in up to 38.5% of HCC patients at the initial diagnosis, while 11.7% of patients with HCC develop bone metastasis after surgical resection of the primary malignancy[4,33,34]. Moreover, the cumulative incidence of bone metastasis 1 year after diagnosis of extrahepatic disease in patients with HCC is 6.4%[35]. Bone metastasis in patients with HCC is most commonly diagnosed in the axial skeleton [vertebral column (up to 40%), pelvic bone, and ribs][31,34,36]. HCC-associated bone metastases are predominantly osteolytic (flake-like or erosion-like decline in bone density), but it could also be presented as osteoblastic metastasis and formation of expansive soft tissue mass[37,38].

Due to the aggressive disease course, studies investigating bone fractures in patients with HCC are very rare. Some data suggest that up to 13.2% of patients with HCC sustain bone fracture[25], but future well-designed large-scale prospective epidemiological studies are needed to analyze the fracture risk in patients with HCC. In cases when fracture risk analysis is not available, clinical surrogate markers of increased bone fragility are used to indirectly assess bone fracture risk. Reduced BMD (obtained by dual-energy X-ray absorptiometry) is widely accepted as a suitable surrogate marker in the clinical assessment of fracture risk[39,40]. According to recommendations by the World Health Organiz-

ation, individuals with a T score in the range between -1 to -2.5 are defined as those with osteopenia, while individuals with a T score lower than -2.5 are diagnosed with osteoporosis[41].

Although osteopenia and osteoporosis are commonly investigated in patients with various forms of chronic liver diseases[42], a recent shift has been directed at investigating HCC-associated BMD alterations (independent of bone metastasis)[26,27,43,44]. Sharma *et al*[43] demonstrated that vertebral BMD reduction, high tumor burden, and older age are important determinants of post-transplantation mortality in individuals with HCC. Miyachi *et al*[26] reported that preoperative low vertebral BMD was an independent risk factor for long-term outcomes after hepatectomy in male patients with HCC but not in female patients with HCC. Arguably, this sex specificity could be explained by the postmenopausal hormonal status of female subjects included in the study[26]. Most recently, Meister *et al*[44] and Müller *et al*[27] demonstrated that low BMD was associated with inferior survival in elderly patients with HCC undergoing partial hepatectomy or transarterial chemoembolization. These studies coherently implied that the integration of vertebral BMD measurement in a novel clinical algorithm could improve survival prediction and clinical management of patients with HCC and that using rehabilitation programs and specific antiresorptive therapy may further improve treatment outcomes among these individuals[26,27,43,44].

The current understanding of skeletal alterations in patients with HCC is limited by the small sample sizes in available retrospective studies as well as a modest number of these studies. Additionally, previous studies were conducted using vertebral BMD derived from multidetector computed tomography scans and not dual-energy X-ray absorptiometry, which is considered the "gold standard" in the clinical assessment of fracture risk. In addition, widely accepted up-to-date clinical methods used to assess skeletal status have certain limitations. For example, BMD is used as a two-dimensional surrogate marker of bone fragility even though it does not account for other intrinsic bone characteristics (bone quality; Figure 1). Moreover, BMD is in the physiological range in the majority of individuals with bone fractures, and anti-osteoporotic therapy has been reported to reduce fracture risk without affecting BMD[42]. Another important factor is the non-uniformity of the human skeleton, indicating that assessment of bone alterations in patients with HCC should be site specific.

Therefore, future studies should focus on resolving these limitations and on utilizing a hierarchical approach in analyzing the contribution of each bone fragility determinant in patients with HCC (Figure 1). The long-term benefit of multiscale and advanced assessment of bone fragility determinants could be creating a new patient-specific diagnostic algorithm that would provide a more accurate clinical assessment of the skeletal status in patients with HCC.

MUSCULAR ALTERATIONS IN PATIENTS WITH HCC

Recently, numerous research teams have begun studying age-related muscular alterations, which play a significant role in the deteriorating health and well-being of elderly individuals[45-47]. Muscular alterations are considered a natural course of aging[45-47], but these alterations could be exacerbated in various chronic comorbidities and malignancies. Among the most frequent muscular abnormalities that are prevalent in multiple tumors, including HCC, are sarcopenia[48-50] and myosteatosis[51,52]. Sarcopenia is a condition characterized by a loss of skeletal muscle mass and deterioration in muscle strength and function, while myosteatosis is characterized by intermuscular and intramuscular accumulation of adipose tissue[52].

A recent systematic review and meta-analysis revealed that the incidence rate of sarcopenia among patients with HCC was 42%[49]. However, there was substantial heterogeneity among the included studies (95% confidence interval: 0.36-0.48)[49]. The data suggested that 30%-40% of patients with HCC that developed from liver cirrhosis showed accelerated progression of sarcopenia at the time of diagnosis[51,53]. Also, the sex-specificity of HCC-associated sarcopenia was revealed in which the prevalence of sarcopenia was higher in studies that included predominantly male patients compared to studies conducted with fewer males (45% *vs* 37%, respectively)[49]. Lastly, the incidence rate of HCC-associated sarcopenia was reported to be higher in patients younger than 60 years when compared to older individuals [49,54]. Thus, previous studies suggest that sarcopenia could be a reliable predictor of inferior outcomes and lower survival rates in patients with HCC[55-59], possibly due to an increased risk of postoperative complications and reduced tolerance to chemotherapy.

Myosteatosis has initially been neglected in previous studies, but research interest in myosteatosis is currently increasing[52,59-62]. Previous data suggested a highly variable prevalence of myosteatosis among individuals with HCC (15.2%-38.8%)[52,60-62]. Patients with HCC-associated myosteatosis had a higher overall mortality rate compared to individuals with HCC who did not have myosteatosis[60]. Moreover, the 5-year cancer-specific survival rate after hepatectomy was significantly worse in individuals with myosteatosis in comparison to patients with HCC who did not have myosteatosis[61]. These studies suggest that myosteatosis could be associated with a reduced post-treatment survival rate in patients with HCC[52,60-62].

The current understanding of muscular alterations in patients with HCC is affected by the limited sample size in the available retrospective studies. These studies reported high variability and heterogeneity in the risk of developing muscular alterations in patients with HCC, suggesting that cautious interpretation of the pooled data is necessary. These studies used different diagnostic criteria when defining sarcopenia and myosteatosis, which indicates that a uniform and standardized diagnostic approach should be applied in future studies. Further, multiple muscles or groups of muscles should be utilized to accurately assess sarcopenia and myosteatosis. Muscle function, rather than muscle mass, could be an additional and powerful predictor that must be investigated in patients with HCC (Figure 1). Therefore, future well-designed clinical studies should focus on resolving these limitations to confirm the benefits of applying early screening and prevention measures (nutritional support and physical exercise). Since individuals with reduced muscle mass and/or

impaired muscle function have a greater risk of bone loss (osteosarcopenia), balance impairments, and fractures[46,63], clinical tools designed to simultaneously improve skeletal and muscle health are warranted in individuals with HCC.

ETIOPATHOGENETIC MECHANISMS LEADING TO MUSCULOSKELETAL ALTERATIONS IN HCC PATIENTS

Etiopathogenetic mechanisms leading to musculoskeletal alterations in patients with HCC are complex and not fully understood. Musculoskeletal alterations in patients with HCC are believed to result from the complex interplay between nutritional deficiencies, physical inactivity, hepatic dysfunction, hormonal/cytokine disruptions, and immunological imbalance (Figure 2), which could result in a loss of bone and muscle mass, impaired bone and muscular quality, bone and muscle tissue disorganization, and impaired musculoskeletal function[46,47,64].

The systemic proinflammatory milieu associated with HCC triggers the release of numerous cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-α, cyclooxygenases, and prostaglandin E2[44,49,65]. Increased concentrations of cytokines play a pivotal role in HCC-induced cachexia, muscle wasting, and bone resorption, perpetuating the musculoskeletal alterations observed in these patients. It is unclear whether the systematic and local HCC-induced proinflammatory environment accelerates bone loss through stimulation of osteoclastogenesis and activation of the Wnt/β-catenin pathway[51,66]. Liver dysfunction disrupts standard metabolic mechanisms and hormonal regulation, causing a decline in the serum concentrations of insulin-like growth factor-1 and sex hormones (especially unbound testosterone), thereby displaying a negative effect on the musculoskeletal system[49].

It has been commonly believed that the interaction between the skeletal and muscular systems is primarily mechanical. However, recent studies have demonstrated that bone and muscle tissues have additional endocrine and paracrine functions enabling complex bidirectional bone-muscle crosstalk[46,47]. Local and systematic effects of bone-muscle crosstalk are the foundation for understanding osteosarcopenia in individuals with various chronic liver diseases[46,47,67,68], including HCC (Figure 2). Muscles release secretory factors known as myokines, that are implicated in positively or negatively affecting the bone independent of mechanical loading. Insulin-like growth factor-1, fibroblast-like growth factor 2, myostatin, irisin, brain-derived neurotrophic factor, osteoglycin, osteoactivin, IL-6, IL-7, and IL-15 are examples of bone-affecting myokines[46,51,69]. Myostatin, IL-6, and follistatin can facilitate the systemic hyperinflammatory state caused by HCC, especially in HCC that developed from advanced liver fibrosis and liver cirrhosis[51,70]. In addition, HCC can affect cellular processes, leading to cell autophagy, oxidative stress, and mitochondrial dysfunction, ultimately leading to musculoskeletal atrophy[49,71].

Currently, there are only a few known bone-derived factors that influence skeletal muscles. Osteokines secreted by osteoblast or osteoclasts and vascular endothelial growth factor derived by bone marrow mesenchymal cells are two examples. Further research into this field is needed[46]. Vascular endothelial growth factor is a crucial angiogenesis-driving factor in the primary HCC lesion as well as in osteolytic bone metastasis[4,24,34]. It has an activating effect on bone resorption through the OPG-RANKL pathway[44,72,73].

Some studies that suggest that the immunological nature of musculoskeletal alterations in HCC are based on poorly understood interactions between the skeletal, muscular, and immune systems and the HCC lesion[44,74]. It is hypothesized that certain anti-resorptive drugs may display significant anti-tumor effects *via* various immunological pathways [44]. It should be noted that changes in cellular metabolism and mitochondrial dysfunction are possible links in cancer-induced cachexia and musculoskeletal alterations[75,76]. These data indicate possible therapeutic value of various factors contributing to cellular oxidative metabolism in patients with HCC-associated musculoskeletal alterations, warranting further research[76,77].

Since understanding multifactorial etiopathogenetic mechanisms responsible for HCC-associated musculoskeletal alterations is still limited, future research should focus on resolving this complex interconnection. These new insights may lead to the development of new cutting-edge therapeutic modalities specifically designed to alleviate the musculoskeletal burden in patients with HCC.

CONCLUSION

Musculoskeletal alterations in HCC, though less common than liver-related complications, can significantly impact the quality of life of patients with HCC. Frequent musculoskeletal alterations associated with HCC are bone metastases, osteosarcopenia, and myosteatosis. However, these complications are frequently overlooked in the clinical management of patients with HCC. Due to the limited data regarding HCC-induced musculoskeletal alterations and its etiopathogenetic mechanisms, further multidisciplinary research on HCC-associated musculoskeletal alterations is needed to provide better clinical management and treatment options and to improve the quality of life in patients with HCC. Considering that individuals with lower bone mass are more likely to present with impaired muscle function and that individuals with impaired muscle function will develop skeletal impairment, clinical tools designed to simultaneously improve skeletal and muscle health are warranted in individuals with HCC.

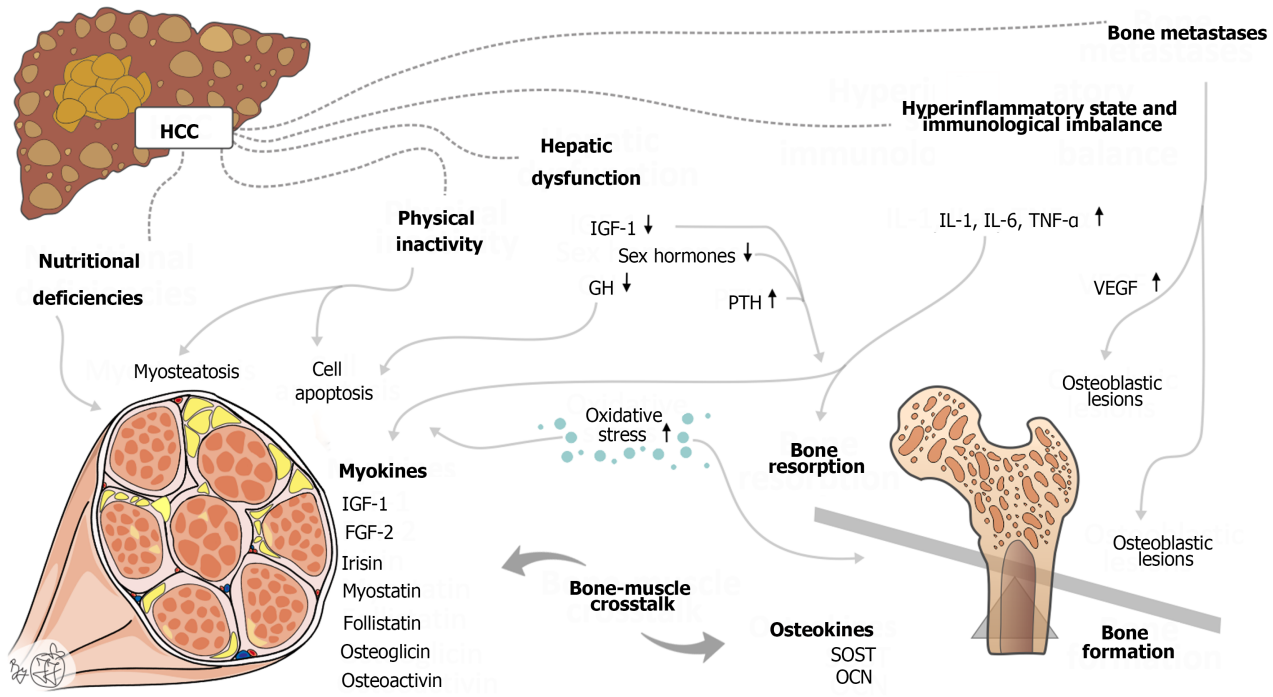

Figure 2 Schematic representation of possible etiopathogenetic mechanisms of musculoskeletal alterations in patients with hepatocellular carcinoma. The possible roles of multiple factors leading to musculoskeletal alterations in patients with hepatocellular carcinoma are shown. HCC: Hepatocellular carcinoma; IGF-1: Insulin-like growth factor 1; FGF-2: Fibroblast-like growth factor 2; GH: Growth hormone; IL: Interleukin; PTH: Parathyroid hormone; OCN: Osteocalcin; SOST: Sclerostin; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor.

FOOTNOTES

Author contributions: Jadzic J conceptualized the study, wrote the initial draft, and conducted the visualization; Jadzic J and Djonic D acquired the data; Djonic D reviewed and edited the manuscript; and all authors read and approved the final version of the manuscript.

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ORIGINAL ARTICLE

Case Control Study

Urgent one-stage endoscopic treatment for choledocholithiasis related moderate to severe acute cholangitis: A propensity score-matched analysis

Yang Zhou, Yin-Qiu Zhang, Shuai-Jing Huang, Yan Liang, Xiao Liang, Masoom Wali, Ya-Dong Feng

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Abstract

BACKGROUND

During emergency endoscopic retrograde cholangiopancreatography (ERCP), the safety and feasibility of performing one-stage endoscopic treatment for patients with acute cholangitis (AC) due to choledocholithiasis are unclear.

AIM

To investigate the safety and feasibility of one-stage endoscopic treatment for moderate to severe AC.

METHODS

We enrolled all patients diagnosed with moderate to severe cholangitis due to common bile duct stones from January 2019 to July 2023. The outcomes were compared in this study between patients who underwent ERCP within 24 h and those who underwent ERCP 24 h later, employing a propensity score (PS) framework. Our primary outcomes were intensive care unit (ICU) admission rates, ICU length of stay, and duration of antibiotic use.

RESULTS

In total, we included 254 patients and categorized them into two groups based on the time elapsed between admission and intervention: The urgent group (≤ 24 h, n = 102) and the elective group (> 24 h, n = 152). Ninety-three pairs of patients with similar characteristics were selected by PS matching. The urgent ERCP group had more ICU admissions (34.4% *vs* 21.5%, P = 0.05), shorter ICU stays (3 d *vs* 9 d, P < 0.001), fewer antibiotic use (6 d *vs* 9 d, P < 0.001), and shorter hospital stays (9 d *vs*

18.5 d, $P < 0.001$). There were no significant differences observed in adverse events, in-hospital mortality, recurrent cholangitis occurrence, 30-d readmission rate or 30-d mortality.

CONCLUSION
Urgent one-stage ERCP provides the advantages of a shorter ICU stay, a shorter duration of antibiotic use, and a shorter hospital stay.

Key Words: Acute cholangitis; Endoscopic retrograde cholangiopancreatography; One-stage treatment; Optimal time

Core Tip: We investigated the safety and feasibility of one-stage endoscopic treatment for moderate to severe acute cholangitis. Our study found that patients who underwent endoscopic retrograde cholangiopancreatography within 24 h had a shorter intensive care unit stay, a shorter duration of antibiotic use, and a shorter hospital stay.

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INTRODUCTION

Acute cholangitis (AC) is a severe and life-threatening infection that affects the biliary tract. It is a significant digestive disorder characterized by rapid onset and is common. Approximately 10%-29% of people with AC develop sepsis[1,2], and approximately 5% of patients progress to septic shock[3]. In severe cases, AC can be fatal. Currently, the Tokyo Guideline 2018 (TG18) criteria are used to diagnose and categorize ACs as mild, moderate, or severe cholangitis[4]. The primary cause of AC is biliary obstruction, which is often caused by cholelithiasis. Approximately 53% of patients with severe AC (SAC) require admission to the intensive care unit (ICU)[5].

While treating SAC, fluid resuscitation and antibiotics need to be administered as initial therapy. In addition, emergent biliary decompression is necessary to improve clinical outcomes[4]. The primary treatment choice for AC is endoscopic retrograde cholangiopancreatography (ERCP), which benefits approximately 90% of patients[6-10]. It is essential to adhere to the principle of "the sooner, the better" when performing ERCP treatment for AC. However, emergency ERCP biliary drainage in patients with severe cholangitis is associated with a significantly high risk of morbidity and mortality. In cases of early ERCP for AC associated with choledocholithiasis, patients with severe cholangitis are frequently subjected to brief procedures such as endoscopic nasobiliary drainage (ENBD) or stenting[9,11,12]. Nevertheless, this additional ERCP procedure not only prolongs the duration of hospital stay but also increases associated risks[12-16]. It is unclear whether single-stage stone removal is feasible for individuals with AC. The optimal timing for ERCP is yet a matter of debate. Therefore, we retrospectively examined and evaluated patients who underwent ERCP for moderate to SAC with choledocholithiasis. The aim was to assess the feasibility and safety of urgent single-stage stone removal for moderate to SAC.

MATERIALS AND METHODS

The study was conducted at Zhongda Hospital Affiliated with Southeast University. The study was approved by the Ethics Committee (2019ZDSYLL094-P01). All methodologies employed in this study strictly adhered to the pertinent guidelines and regulations.

Inclusion criteria

We collected data from the endoscopic reporting system for all patients who underwent ERCP procedures following admission to the emergency department between January 2019 and July 2023. The inclusion criteria were as follows: (1) Diagnosed with AC in accordance with the TG13 or TG18[17,18]; (2) Aged > 18 years; and (3) Willing to undergo ERCP.

Exclusion criteria

The exclusion criteria were as follows: (1) Had mild AC; (2) Did not undergo endoscopic retrograde lithotomy; and (3) Had non-common bile duct (CBD) stones detected *via* cholangiopancreatography.

Data records

We retrieved data for all emergency ERCP procedures performed from January 2019 to July 2023 from the endoscopy reporting system, all patients were diagnosed with moderate to severe cholangitis due to CBD stones. The flowchart of this study is listed as Figure 1. The patients in the study were categorized into two groups based on the time between admission and intervention. These patients were classified into urgent (≤ 24 h, n = 102) and elective (> 24 h, n = 152) ERCP groups. The time span from admission to intervention was considered the time between registration in the emergency room and ERCP. We then sorted and reviewed patient demographic data, presenting symptoms, and ERCP outcomes. These data included the date, time between symptom onset and ERCP, admission and ERCP procedures, as well as laboratory data upon admission, such as the white blood cell (WBC) count, platelets (PLT), total bilirubin (TB), international normalized ratio (INR), creatinine (Cr), serum albumin, C-reactive protein (CRP), and neutrophil/lymphocyte ratio. The time span for biliary drainage was calculated as the duration between admission and the ERCP procedure. Furthermore, postoperative follow-up data were acquired through outpatient examinations or postoperative telephone follow-ups conducted after discharge. Disease severity was graded using the TG18 severity scale[18].

Procedure

Prior to ERCP, we monitored the patients' vital signs, established intravenous access, and administered empiric antibiotic therapy with third-generation cephalosporins. In instances where patients exhibited postshock symptoms, the execution of ERCP was postponed until their condition improved. Urgent ERCP was considered the primary treatment for patients who did not respond to drug therapy or who had moderate to severe disease. Prior to commencing the procedure, all participants provided informed consent. ERCP procedures were performed under general anesthesia and supervised by an anesthesiologist. During ERCP, the maternal endoscope used in this procedure was a therapeutic duodenoscope (Olympus TJF-260, Tokyo, Japan).

In the initial step, we established biliary access. Conventional biliary cannulation was attempted by using a sphincterotome (Microtech, Nanjing, China) and a 0.035-inch guidewire (Microtech, Nanjing, China). Successful biliary access was confirmed by observing visible bile aspiration, and bile samples were extracted for bacterial cultivation upon the manifestation of turbid bile flow. Subsequently, a 3-mm endoscopic sphincterotomy (EST) combined with endoscopic papillary balloon dilation was performed to establish a proper biliary orifice. The balloon was gradually inflated with 0.9% saline solution to the proposed pressure or until the biliary wall could be seen. For the extraction of stones, we employed either a basket or a balloon; for larger stones, mechanical lithotripsy was employed at the discretion of the endoscopist. In cases of cannulation failure, percutaneous transhepatic biliary drainage was explored as an alternative therapeutic option. Subsequently, a nasal biliary drainage tube was placed, and bile acid samples were collected for bacterial culture on postoperative days 1 and 2. The decision to drain the nasal biliary tube was contingent upon the patient's clinical condition. Adverse reactions after drainage were classified according to the ASGE dictionary[19] and included post-ERCP pancreatitis (PEP), bleeding, and infection, among others.

Statistical analysis

The quantitative parameters are reported as either the mean (with range) or median (with interquartile range), depending on the distribution. Categorical variables are presented as the frequency and percentage. The propensity score (PS) framework was used to compare the clinical endpoints of ERCP within 24 h of onset and 24 h after onset. The PS method was used to create a new dataset in which the probability of ERCP occurring within 24 h of or after its occurrence was equal (as in a purely randomized trial) to balance the baseline characteristics of patients. First, multivariate logistic regression was used to predict the probability of ERCP within 24 h (*i.e.*, estimated PS), controlling for the following prespecified covariates: Sex, age, Charlson Comorbidity Index (CCI) score, previous discharge ERCP, history of gallbladder surgery, TB, albumin, Cr, the INR, the PLT, the WBC, and the Tokyo score. The 1:1 nearest neighbor matching algorithm was used to match the two groups (urgent group and elective group) without substitution, and the caliper was 0.2[20] of the PS standard deviation of the logit score. The clinical endpoints were subsequently compared between the two groups in the matched datasets. Statistical analysis, including the χ^2 test, one-way analysis of variance (ANOVA) and multivariate linear regression, was performed using the Statistical Package for Social Sciences (SPSS, Inc., version 27.0 for Windows, Chicago, IL, United States). A P value of < 0.05 was considered to indicate statistical significance.

RESULTS

Population characteristics

From January 2019 to July 2023, a total of 456 patients with acute cholangitis were screened. Among these, 74, 16, 19 and 16 patients were excluded due to mild acute cholangitis, refusal of endoscopic treatment, upper gastrointestinal anatomy changes, and preference of PTBD or PTGBD, respectively. Additionally, 19, 20, 12 and 26 patients were excluded due to intrahepatic stone, acute acalculous cholecystitis, dysfunction of previous biliary stents and malignant obstructions, respectively. Consequently, 254 patients were included, 102 (40.2%) of whom underwent ERCP within 24 h of presentation and 152 (59.8%) after 24 h. The mean age was 69.47 (± 15.81) years, 47.6% were male, and 100% had choledocholithiasis-related cholangitis. The mean CCI score was 1 (0-7), and ERCP was performed for a mean time span of 48 (1-312) h. Cholangitis severity was categorized per Tokyo guidelines: Score 1 = 0%, score 2 = 72%, and score 3 = 28%. Table 1 shows the baseline patient characteristics before and after PS matching. After PS matching, 93 pairs of patients with similar traits were selected (Algorithm 1, Table 1). The proportion of patients who underwent one-step stone

Table 1 Characteristics of the study population and endoscopic retrograde cholangiopancreatography procedures

	Before matching				After matching			
	Total, *n* = 254	ERCP ≤ 24 h, *n* = 102	ERCP > 24 h, *n* = 152	*P* value	Total, *n* = 186	ERCP ≤ 24 h, *n* = 93	ERCP > 24 h, *n* = 93	*P* value
Age, yr	69.47 ± 15.81	70.73 ± 15.24	68.63 ± 16.18	0.362	70.32 ± 15.39	71.05 ± 15.26	69.58 ± 15.56	0.515
Male sex, *n* (%)	121 (47.6)	58 (56.9)	63 (41.4)	0.016	94 (50.5)	54 (58.1)	40 (43)	0.04
CCI	1 (0-7)	1 (0-5)	1 (0-7)	0.108	1 (0-7)	1 (0-3)	1 (0-7)	0.187
Past medical history								
ERCP, *n* (%)	30 (11.8)	13 (12.7)	17 (11.2)	0.706	22 (11.8)	13 (14)	9 (9.7)	0.364
Cholecystectomy, *n* (%)	67 (26.4)	27 (26.5)	40 (26.3)	0.978	44 (23.7)	22 (23.7)	22 (23.7)	1
Lab values								
WBC count as/μL	10.32 ± 6.71	12.57 ± 6.61	8.81 ± 6.37	< 0.001	10.58 ± 7.03	12.11 ± 6.44	9.06 ± 7.3	0.003
Platelet count as/μL	173.96 ± 71.08	164.21 ± 73.35	180.5 ± 68.99	0.594	168.11 ± 71.33	164.85 ± 74.65	171.38 ± 68.09	0.534
CRP in mg/L	75.17 ± 76.03	94.9 ± 79.32	61.75 ± 70.95	< 0.001	78.53 ± 76.6	89.66 ± 76.37	76.37 ± 75.75	0.079
NLR (%)	7.94 (0.81-106.31)	15.67 (1.36-106.31)	6.9 (0.805-64.13)	< 0.001	18.545 (2.55-64.13)	19.87 (11.2)	16.42 (2.55-64.13)	< 0.001
INR	1.2 ± 0.22	1.23 ± 0.97	1.14 ± 0.92	< 0.001	1.21 ± 0.23	1.25 ± 0.27	1.17 ± 0.17	0.012
D2 polymers	657 (0.21-26652)	1567 (76-26652)	504 (0.38-15502)	< 0.001	1193.5 (479-15502)	1455 (479-4811)	832 (504-15502)	0.005
Creatinine in mg/dL	0.826 (0.34-8.32)	1.01 (0.34-5.86)	0.76 (0.44-8.32)	< 0.001	1.10 (0.77-5.86)	1.15 (0.77-5.86)	1.02 (0.79-3.1)	0.013
TB in mg/dL	2.61 (0.28-22.52)	3.7 (0.29-15.02)	2.14 (0.28-22.52)	0.021	3.58 ± 2.98	3.78 ± 2.8	3.38 ± 3.14	0.363
AST in U/L	108.5 (13-4051)	128.5 (13-744)	104 (15-4051)	0.168	118 (40-539)	131 (40-497)	116 (46-539)	0.53
ALT in U/L	201.68 ± 206.61	196.67 ± 173.10	205.03 ± 226.84	0.652	222 (68-512)	259 (68-512)	209 (88-479)	0.55
γ-GT in U/L	406.66 ± 354.98	425.8 ± 376.89	393.82 ± 340.16	0.463	383.61 ± 332.61	405.97 ± 347.74	361.25 ± 361.25	0.361
Albumin in g/dL	35.8 (15.9-46.6)	35.9 (15.9-48.7)	37.8 (25.1-49.1)	0.033	33.7 (24.3-36.1)	32.6 (24.3-35.7)	33.8 (25.4-36.1)	0.163
Tokyo Score								
3	48 (28)	39 (38.2)	32 (21.1)	0.003	58 (31.2)	35 (37.6)	23 (24.7)	0.058
2	206 (72)	63 (61.8)	120 (78.9)		128 (68.8)	58 (62.4)	70 (75.3)	
ERCP procedure								
Door to ERCP time in h	48 (1-312)	8.5 (1-24)	120 (27-312)	< 0.001	25.5 (1-312)	9 (1-24)	120 (27-312)	< 0.001
ERCP procedure time (min)	60 (25-780)	60 (30-200)	60 (30-335)	0.714	60 (26-780)	60 (30-200)	60 (30-335)	0.52
One-stage ERCP, *n* (%)	254 (100)	102 (100)	152 (100)	1	186 (100)	93 (100)	93 (100)	1
CBD, *n* (%)	254 (100)	102 (100)	152 (100)	1	186 (100)	93 (100)	93 (100)	1
Stones size (mm)	8 (2-25)	9 (2-25)	8 (2-25)	0.222	8 (2-25)	9 (2-25)	8 (2-25)	0.368
Multiple stones, *n* (%)	109 (42.9)	35 (34.3)	74 (48.7)	0.023	79 (42.5)	32 (34.4)	47 (50.5)	0.026
Common bile duct width (mm)	13 (4-33)	14 (4-33)	14 (5-33)	0.016	13 (4-33)	13 (4-33)	12.1 (5-25)	0.038
EST, *n* (%)	177 (69.7)	75 (73.5)	102 (67.1)	0.275	128 (68.8)	69 (74.2)	59 (63.4)	0.113
EPBD, *n* (%)	204 (80.3)	90 (88.2)	114 (75)	0.009	149 (80.1)	83 (89.2)	66 (71)	0.002
Pancreatic stent placement, *n* (%)	21 (8.3)	5 (4.9)	16 (10.5)	0.111	15 (8.1)	5 (5.4)	10 (10.8)	0.178

Nasal Biliary Drainage Catheter placement, *n* (%)	251 (98.8)	100 (98)	151 (99.3)	0.346	183 (98.4)	91 (97.8)	92 (98.9)	0.561
HLL, *n* (%)	21 (8.3)	11 (10.8)	10 (6.6)	0.233	16 (8.6)	11 (11.8)	5 (5.4)	0.117

SMD: Standardized mean difference; CCI: Charlson Comorbidity Index; ERCP: Endoscopic retrograde cholangiopancreatography; WBC: White blood cell; CRP: C-reactive protein; NLR: Neutrophil-lymphocyte ratio; INR: International normalized ratio; TB: Total bilirubin; AST: Aspartate transaminase; ALT: Alanine transaminase; γ-GT: γ-glutamyl transpeptidase; CBD: Common bile duct stones; LC-IntraERCP: Laparoscopic cholecystectomy combined with intraoperative endoscopic retrograde cholangiopancreatography; EST: Endoscopic sphincterotomy; EPBD: Endoscopic papillary balloon dilatation; HLL: Holmium Laser Lithotripsy.

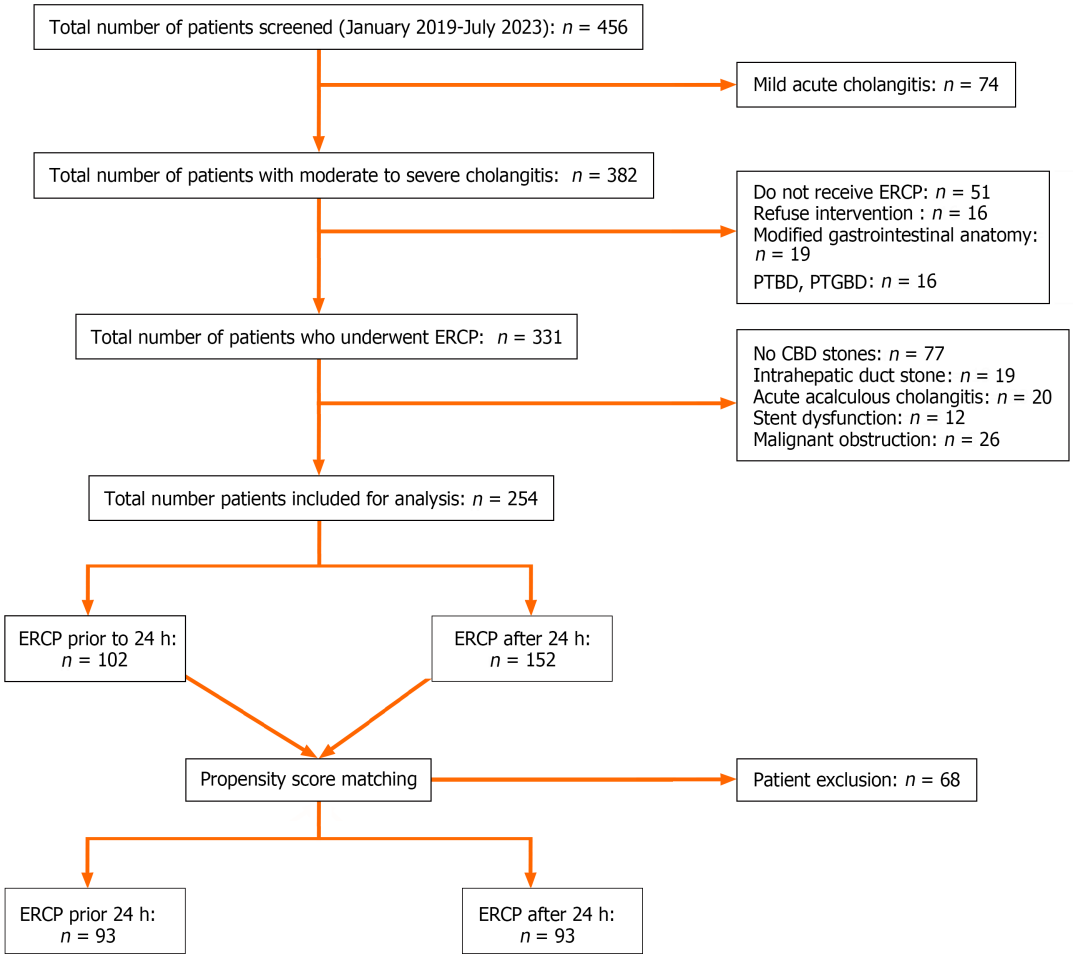

Figure 1 Flow diagram of patients' selection. ERCP: Endoscopic retrograde cholangiopancreatography; PTBD: Percutaneous transhepatic biliary drainage; PTGBD: Percutaneous transhepatic gallbladder drainage; CBD: Common bile duct.

extraction after matching was consistent (100% *vs* 100%, $P = 1$) (Table 1).

Primary clinical outcomes

Our primary outcome was ICU admission rate, ICU length of stay, and duration of antibiotic use (Table 2). The results derived from our analysis of a PS-matched population indicated a significant difference in ICU admission rates between the urgent ERCP group and the elective ERCP group (34.4% *vs* 21.5%, $P = 0.05$). Importantly, there was a significant difference in ICU stay length between the urgent ERCP and elective ERCP groups, with the urgent group having a shorter stay (3 d *vs* 9 d, $P < 0.001$). Additionally, compared with those in the elective group, the patients in the urgent ERCP group had a shorter duration of antibiotic use (6 d *vs* 9 d, $P < 0.001$). Univariate linear regression analysis of ICU stay length revealed independent correlations with variables, including WBC [95% confidence interval (CI): 0.18-0.82, $P = 0.003$], CRP (95%CI: 0.01-0.08, $P = 0.015$), Cr (95%CI: 4.22-8.28, $P < 0.001$), age (95%CI: -0.66 to -0.07, $P = 0.016$), and the time span of ERCP (hours) (95%CI: 0.04-0.06, $P < 0.001$). Additionally, ICU stay length was not significantly correlated with one-stage endoscopic treatment, EST, ENBD, adverse events, 30-d readmission, or recurrent cholangitis (Table 3). Multivariate linear regression analysis of the matched data revealed significant correlations between ERCP delay time (95%CI: 0.03-0.06, $P < 0.001$), Cr level (95%CI: 0.07-3.56, $P = 0.041$), and ICU stay length.

Table 2 Outcomes of endoscopic retrograde cholangiopancreatography

	Before matching				After matching			
	Total, *n* = 254	ERCP ≤ 24 h, *n* = 102	ERCP > 24 h, *n* = 152	*P* value	Total, *n* = 186	ERCP ≤ 24 h, *n* = 93	ERCP > 24 h, *n* = 93	*P* value
ERCP intervention type, *n* (%)								
Complete stone removal	250 (98.4)	101 (99)	149 (98)	0.533	184 (98.9)	92 (98.9)	92 (98.9)	1
Biliary stent insertion	4 (1.6)	1 (1)	3 (2)	0.533	2 (1.1)	1 (1.1)	1 (1.1)	1
Technical success rate, *n* (%)	250 (98.4)	101(99)	149 (98)	0.533	183 (98.4)	92 (98.9)	91 (98)	0.561
ERCP failure, *n* (%)	4 (1.6)	1 (1)	3 (2)	0.533	3 (1.6)	1 (1.1)	2 (2.2)	1
Duration of antibiotic use (d)	7 (1-28)	6 (2-15)	8 (2-26)	< 0.001	7 (2-28)	6 (2-18)	9 (2-28)	< 0.001
In-hospital mortality, *n* (%)	3 (1.2)	0	3 (2)	0.153	2 (1.1)	0	2 (2.2)	0.155
30-d mortality, *n* (%)	7 (2.8)	2 (2)	5 (3.3)	0.526	5 (2.7)	2 (2.2)	3 (3.2)	0.65
Recurrent cholangitis, *n* (%)	7 (2.8)	3 (2.9)	4 (2.6)	0.883	6 (3.2)	3 (3.2)	3 (3.2)	1
LOHS, (d)	10 (3-71)	9 (3-39)	18 (5-71)	< 0.001	9 (3-71)	9 (3-39)	18.5 (7-71)	< 0.001
Required ICU stay, *n* (%)	61 (24)	33 (32.4)	28 (18.4)	0.011	52 (28)	32 (34.4)	20 (21.5)	0.05
ICU stay length, (d)	9 (1-71)	3 (1-15)	8 (1-71)	0.003	4.5 (1-71)	3 (1-15)	9 (1-71)	< 0.001
30 d readmission, *n* (%)	33 (13)	15 (14.7)	18 (11.8)	0.506	29 (15.6)	14 (15.1)	15 (16.1)	0.84
ERCP-related complications, *n* (%)	42 (16.5)	18 (17.7)	24 (15.8)	0.696	29 (15.6)	16 (17.2)	13 (14)	0.544
PEP	23 (9.1)	9 (8.8)	14 (9.2)	0.916	17 (9.1)	9 (9.7)	8 (8.6)	0.799
Cholangitis	9 (3.5)	6 (5.9)	3 (2)	0.099	7 (3.8)	5 (5.4)	2 (2.2)	0.248
Bleeding	6 (2.4)	4 (3.9)	2 (1.3)	0.18	4 (2.2)	3 (3.2)	1 (1.1)	0.312
Others	2 (0.8)	2 (2)	0	0.083	2 (1.1)	2 (2.2)	0	0.155

ERCP: Endoscopic retrograde cholangiopancreatography; LOHS: Length of hospital stay; PEP: Post-endoscopic retrograde cholangiopancreatography pancreatitis; ICU: Intensive care unit.

Secondary clinical outcomes

According to our analysis of the PS-matched population (Table 2), the length of hospital stay (LOHS) in the urgent group was significantly shorter than that in the elective group (9 d *vs* 18.5 d, $P < 0.001$). The two groups exhibited no significant differences in 30-d readmission (15.1% *vs* 16.1%, $P = 0.84$), recurrent cholangitis (2.9% *vs* 2.6%, $P = 0.883$), in-hospital mortality (0% *vs* 2.2%, $P = 0.155$), 30-d mortality (2.2% *vs* 3.2%, $P = 0.65$), adverse events after ERCP (17.65% *vs* 15.79%, $P = 0.696$), PEP (8.82% *vs* 9.21%, $P = 0.916$), bleeding (3.9% *vs* 1.3%, $P = 0.180$), biliary tract infection (5.9% *vs* 1.97%, $P = 0.099$), or other ERCP-related adverse events (1.96% *vs* 0, $P = 0.083$).

Subgroup analysis of patients with SAC

After PS matching (Table 4), 58 patients in the cohort presented with severe biliary tract infection according to a Tokyo score of 3. Among these patients, 60.3% underwent ERCP within 24 h of onset, while 39.7% underwent ERCP after 24 h. Subsequently, we compared outcomes between the urgent ERCP group and the elective ERCP group within the subset of patients who experienced severe cholangitis. No significant difference in ICU admission rates was observed between the two groups (60% *vs* 47.8%, $P = 0.362$). The urgent group had a significantly shorter ICU stay than did the elective group (4 d *vs* 11 d, $P = 0.014$), a significantly shorter duration of antibiotic use (17.1% *vs* 17.4%, $P = 0.98$), and a markedly shorter LOHS (9 d *vs* 20 d, $P < 0.001$). Additionally, within 30 d, there were no significant differences between the two subgroups in terms of readmission (17.1% *vs* 17.4%, $P = 0.98$), in-hospital mortality (0% *vs* 4.3%, $P = 0.213$), 30-d mortality (5.7% *vs* 8.7%, $P = 0.661$), occurrence of adverse events after ERCP (22.86% *vs* 13.04%, $P = 0.351$), PEP (8.57% *vs* 4.35%, $P = 0.535$), bleeding (2.86% *vs* 4.35%, $P = 0.761$), biliary tract infection (8.57% *vs* 0%, $P = 0.149$), or occurrence of other ERCP-related adverse events (2.86% *vs* 0, $P = 0.414$).

Table 3 Linear regression analyses to assess intensive care unit length of stay

	Univariate analysis		Multivariate analysis	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
WBC count	0.503 (0.185 to 0.821)	0.003	-0.092 (-0.305 to 0.12)	0.387
Platelet count	-0.031 (-0.069 to 0.007)	0.105		
CRP	0.046 (0.009 to 0.082)	0.015	0.002 (0.021 to 0.024)	0.877
NLR	-0.006 (-0.181 to 0.169)	0.945		
INR	-4.986 (-15.932 to 5.961)	0.365		
TB	-0.101 (-1.26 to 1.058)	0.862		
Cr	6.248 (4.216 to 8.281)	< 0.001	1.818 (0.073 to 3.564)	0.042
Albumin	0.569 (0.043 to 1.095)	0.035	0.02 (-0.308 to 0.347)	0.905
ALT	-0.005 (-0.017 to 0.007)	0.375		
AST	-0.001 (-0.007 to 0.005)	0.789		
Multiple stones	-3.31 (-9.87 to 3.249)	0.316		
CCI	1.466 (-0.713 to 3.644)	0.183		
Age	-0.367 (-0.663 to -0.072)	0.016	-0.086 (-0.256 to 0.083)	0.312
Severity of AC	3.188 (-3.434 to 9.809)	0.338		
Time to ERCP	0.051 (0.340 to 0.059)	< 0.001	0.044 (0.033 to 0.056)	< 0.001
Common bile duct width	-0.002 (-0.34 to 0.335)	0.988		

OR: Odds ratio; CI: Confidence interval; CCI: Charlson Comorbidity Index; ERCP: Endoscopic retrograde cholangiopancreatography; WBC: White blood cell; CRP: C-reactive protein; NLR: Neutrophil-lymphocyte ratio; INR: International normalized ratio; TB: Total bilirubin; Cr: Creatinine; AST: Aspartate transaminase; ALT: Alanine transaminase; AC: Acute cholangitis.

Table 4 Outcomes of endoscopic retrograde cholangiopancreatography in the propensity matched population (Tokyo score 3 subgroup)

Patients with Grade III AC	Total, *n* = 58	ERCP ≤ 24 h, *n* = 35	ERCP > 24 h, *n* = 23	*P* value
Duration of antibiotic use (d)	8 (3-28)	7 (3-15)	11 (3-28)	0.004
In-hospital mortality, *n* (%)	1 (1.7)	0	1 (4.3)	0.213
30-d mortality, *n* (%)	4 (6.9)	2 (5.7)	2 (8.7)	0.661
Recurrent cholangitis, *n* (%)	4 (6.9)	2 (5.7)	2 (8.7)	0.661
LOHS, (d)	13 (6-71)	9 (6-17)	20 (14-71)	< 0.001
Required ICU stay, *n* (%)	32 (55.2)	21 (60)	11 (47.8)	0.362
ICU stay length, (d)	6 (1-71)	4 (1-15)	11 (1-71)	0.014
30 d readmission, *n* (%)	10 (17.2)	6 (17.1)	4 (17.4)	0.98
ERCP-related complications, *n* (%)	11 (19)	8 (22.9)	3 (13)	0.351
PEP	4 (6.9)	3 (8.6)	1 (4.3)	0.535
Cholangitis	3 (5.2)	3 (8.6)	0	0.149
Bleeding	2 (3.4)	1 (2.9)	1 (4.3)	0.761
Others	1 (1.7)	1 (2.9)	0	0.414

AC: Acute cholangitis; ERCP: Endoscopic retrograde cholangiopancreatography; LOHS: Length of hospital stay; PEP: Post-endoscopic retrograde cholangiopancreatography pancreatitis; ICU: Intensive care unit.

DISCUSSION

Numerous studies have been conducted to determine the best timing for biliary decompression in patients with AC. However, the advantages of urgent one-stage endoscopic procedures *via* ERCP for treating moderate to severe cholangitis associated with CBD stones still need further clarification[20-23]. We comprehensively analyzed the characteristics and diagnostic findings of 254 patients diagnosed with AC who were admitted to Zhongda Hospital of Southeast University over the past four years. Within our PS-matched population, multivariate regression analysis was used to identify independent predictors of ICU stay length, including preoperative Cr levels and delay in performing ERCP. Notably, elective ERCP was associated with a longer duration of ICU stay (3 d *vs* 8 d, $P < 0.001$) and a prolonged course of antibiotic treatment (6 d *vs* 9 d, $P < 0.001$). Additionally, elective ERCP resulted in an increased LOHS (9 d *vs* 18.5 d, $P < 0.001$). Similar findings were observed in the unadjusted cohort analysis: ICU stay length (3 d *vs* 8 d, $P = 0.003$), antibiotic duration (6 d *vs* 8 d, $P < 0.001$), and LOHS (9 d *vs* 18 d, $P < 0.001$).

Our investigation concentrated on patients who underwent single-stage endoscopic procedures for AC. In our PS-matched population, the mortality rate was 2.7%. This figure aligns with the findings reported by Park *et al*[12] and Zhang *et al*[14]. Notably, our observation rate was lower than the 5%-11% range documented in other studies[11,21]. One plausible rationale for this variance may stem from the fact that all subjects in our study exclusively underwent single-stage endoscopic procedures, potentially contributing to the observed lower mortality rate. Notably, single-stage endoscopic procedures exhibit both safety and efficacy in addressing biliary drainage and CBD stone clearance in individuals with AC. Previous studies have revealed that one-stage endoscopic treatment has a high cure rate and low complication rate in patients with mild to moderate cholangitis. In a multicenter retrospective study conducted by our team in 2019, the safety and efficacy of this approach were reaffirmed, particularly in patients with severe complications [14]. Eto *et al*[24] also reported a cure rate of 90% within 4 d of single-stage treatment for AC (45 out of 50 patients), as well as complete stone clearance achieved in all patients and a complication rate of only 10% (5 out of 50 individuals). This approach effectively reduces the risks associated with two-stage ERCP procedures. Our study included 254 patients who underwent urgent single-stage endoscopic procedures, all of which resulted in complete stone clearance and a low complication rate of 16.5%. These results suggested that single-stage treatment can be an effective and safe method for treating moderate to SAC associated with stone removal.

In 2023, Hedjoudje *et al*[22] conducted an analytical study based on a substantial database that included 85 patients with severe cholangitis. These patients underwent drainage within 24 h, while the remaining 51 patients underwent drainage 24 h later. The study revealed that the elective ERCP procedure was linked to higher mortality rates (13.0% *vs* 45.5%, $P < 0.001$), prolonged length of ICU stays (4.61 d *vs* 7.41 d, $P = 0.004$), and increased LOHS. In a retrospective study conducted by Muangkaew *et al*[25], a cohort of patients diagnosed with acute biliary pancreatitis associated with cholangitis was analyzed. Of these, 67 out of 95 patients underwent drainage within 72 h. The study revealed no statistically significant differences in mortality, ERCP-related complications, or disease-related complications between the early and elective ERCP groups. However, the early ERCP (< 72 h) group had a shorter LOHS (6.3 ± 4.4 d) than did the elective ERCP group (9.8 ± 6.1 d; $P = 0.002$). The difference in mortality outcomes between the two studies may be attributed to the study of Hedjoudje *et al*[22], patients specifically with severe cholangitis were enrolled, which potentially resulted in significantly greater mortality rates than those in the study of Muangkaew *et al*[25]. This discrepancy may partially explain the differences in mortality outcomes between the two studies.

Given the relatively low mortality rate observed among cholangitis patients in our study, our primary outcome measures included the ICU admission rate, ICU length of stay, and duration of antibiotic use. After analyzing multiple factors within our matched cohort, we found that for every hour of delay in ERCP, patients' ICU stay increased by 0.033 d. Such a prolonged ICU stay not only contributes to increased hospital expenses but also amplifies the risks of hospital-acquired infections and associated adverse events. Our research underscores the imperative for urgent ERCP in patients experiencing moderate to SAC. The delay in receiving ERCP correlates with extended hospital and ICU stays, aligning with findings from prior investigations[11,21,22,25,26]. Nevertheless, we observed a heightened ICU admission rate in the urgent ERCP group, potentially attributed to the greater prevalence of severe cases in that cohort (34.4% *vs* 21.5%, $P = 0.05$). After surgery, medical practitioners typically move patients with severe biliary tract inflammation to the ICU for stabilization. Contrary to this norm, our study demonstrated that patients receiving urgent ERCP exhibited a shorter ICU stay (3 d *vs* 8 d, $P < 0.001$), with no discernible differences in post-ERCP prognostic indicators between the two groups. Despite a higher percentage of severe patients in the urgent group, patients in this subset recovered faster post surgery. Additionally, we assessed the duration of antibiotic usage among patients who underwent ERCP. Patients in the urgent group had a significantly shorter duration of antibiotic usage than did those in the nonurgent group (7 d *vs* 16 d, $P < 0.001$). Simultaneously, our results indicate a reduction in overall hospitalization within the urgent group. These findings collectively affirm the quicker postoperative recovery observed in the urgent group. Furthermore, our multifactorial linear analysis of ICU stay length revealed that Cr levels had a significant impact on ICU stay.

There are several limitations to our research. First, there may be inherent selection bias present, and the results of our research may only reflect the clinical situation within our facility because this was a retrospective single-center study. Second, we implemented strict inclusion and exclusion criteria, which led to a relatively small sample size. To address these issues, further large-scale clinical studies are necessary to confirm our findings.

CONCLUSION

To summarize, urgent one-stage endoscopic treatment is feasible and safe for patients with moderate to SAC. Our

research also showed that if ERCP is performed more than 24 h after admission for moderate to SAC, it may lead to longer stays in the ICU and hospital.

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FOOTNOTES

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ORIGINAL ARTICLE

Retrospective Study

Computed tomography-based radiomics to predict early recurrence of hepatocellular carcinoma post-hepatectomy in patients background on cirrhosis

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Abstract

BACKGROUND

The prognosis for hepatocellular carcinoma (HCC) in the presence of cirrhosis is unfavourable, primarily attributable to the high incidence of recurrence.

AIM

To develop a machine learning model for predicting early recurrence (ER) of post-hepatectomy HCC in patients with cirrhosis and to stratify patients' overall survival (OS) based on the predicted risk of recurrence.

METHODS

In this retrospective study, 214 HCC patients with cirrhosis who underwent curative hepatectomy were examined. Radiomics feature selection was conducted using the least absolute shrinkage and selection operator and recursive feature elimination methods. Clinical-radiologic features were selected through univariate and multivariate logistic regression analyses. Five machine learning methods were used for model comparison, aiming to identify the optimal model. The model's performance was evaluated using the receiver operating characteristic curve [area under the curve (AUC)], calibration, and decision curve analysis. Additionally, the Kaplan-Meier (K-M) curve was used to evaluate the strati-

fication effect of the model on patient OS.

RESULTS

Within this study, the most effective predictive performance for ER of post-hepatectomy HCC in the background of cirrhosis was demonstrated by a model that integrated radiomics features and clinical-radiologic features. In the training cohort, this model attained an AUC of 0.844, while in the validation cohort, it achieved a value of 0.790. The K-M curves illustrated that the combined model not only facilitated risk stratification but also exhibited significant discriminatory ability concerning patients' OS.

CONCLUSION

The combined model, integrating both radiomics and clinical-radiologic characteristics, exhibited excellent performance in HCC with cirrhosis. The K-M curves assessing OS revealed statistically significant differences.

Key Words: Machine learning; Radiomics; Hepatocellular carcinoma; Cirrhosis; Early recurrence; Overall survival; Computed tomography; Prognosis; Risk factor; Delta-radiomics

Core Tip: Hepatocellular carcinoma (HCC) ranks as the sixth most prevalent tumour and stands as the third leading cause of cancer-related deaths globally. In contrast to individuals with HCC in normal liver tissue, those with HCC in the context of cirrhosis frequently experience a higher recurrence rate. Therefore, a machine learning model aimed at predicting the early recurrence of post-hepatectomy HCC in patients with cirrhosis was developed. The study also aimed to stratify patients' overall survival based on the predicted risk of recurrence.

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INTRODUCTION

Hepatocellular carcinoma (HCC) stands as the sixth most prevalent tumour and the third leading cause of cancer-related deaths globally[1]. Liver cirrhosis constitutes the primary risk factor for HCC, affecting nearly 90% of patients with HCC to varying degrees[1,2]. Curative-intent hepatectomy remains the preferred treatment for patients with early HCC[3]. However, the recurrence rate among patients with HCC surpasses 50%, significantly reducing long-term survival rates after resection[4]. The heightened risk of recurrence after curative-intent hepatectomy can be attributed to two patterns: recurrence stemming from residual microvascular metastasis after liver resection and recurrence due to the underlying liver carcinogenicity background[5]. Compared to patients with HCC in normal liver tissue, those with cirrhosis background HCC tend to experience both modes of recurrence, resulting in a higher recurrence rate after resection. Sasaki *et al*[6] observed a consistently higher risk of recurrence in the early postoperative period among patients with cirrhosis, with a 6% higher annual risk compared to non-cirrhotic resection for HCC (15%). Numerous staging systems, such as the Barcelona Clinic Liver Cancer staging system and the albumin-bilirubin grade, have been introduced for assessing the prognosis of patients with HCC[7]. However, these staging systems are more suited for evaluating liver function or guiding therapy. Given the high heterogeneity of HCC, patients with HCC during the same period might exhibit varying prognoses[8]. Currently, there is a lack of individualised prognosis assessment for patients, emphasising the crucial need for systematic surveillance of HCC recurrence and the accurate prediction of recurrence in patients with HCC.

In recent years, numerous studies have confirmed the impact of the inflammatory status within the tumour microenvironment on the occurrence and progression of tumours. However, the invasive nature of diagnosing these biomarkers, with associated risks of tumour seeding, bleeding, and sampling errors, has led to current guidelines not recommending biopsy diagnosis for HCC[9]. Therefore, there is a critical need for a non-invasive HCC marker to predict the early recurrence (ER) of HCC. Radiomics, a technique involving the extraction of quantitative image features through non-invasive, high-throughput analysis of standard medical imaging, presents a promising avenue. This method enables the extraction of data, which can be applied to enhance the accuracy of diagnostic, prognostic, and predictive assessments, ultimately serving as a bridge between medical imaging and individualised medical treatment[10,11]. The specific study flow of radiomics is illustrated in Figure 1.

Delta-radiomics involves extracting radiomics features from the same region of interest in a given patient to examine changes in radiomics characteristics over time. Unlike traditional radiomics methods that use single-phase images for feature extraction, which overlooks the image feature changes induced by alterations in blood flow after the introduction of a contrast agent, delta-radiomics offers a more comprehensive reflection of changes in pathological tissue or blood flow

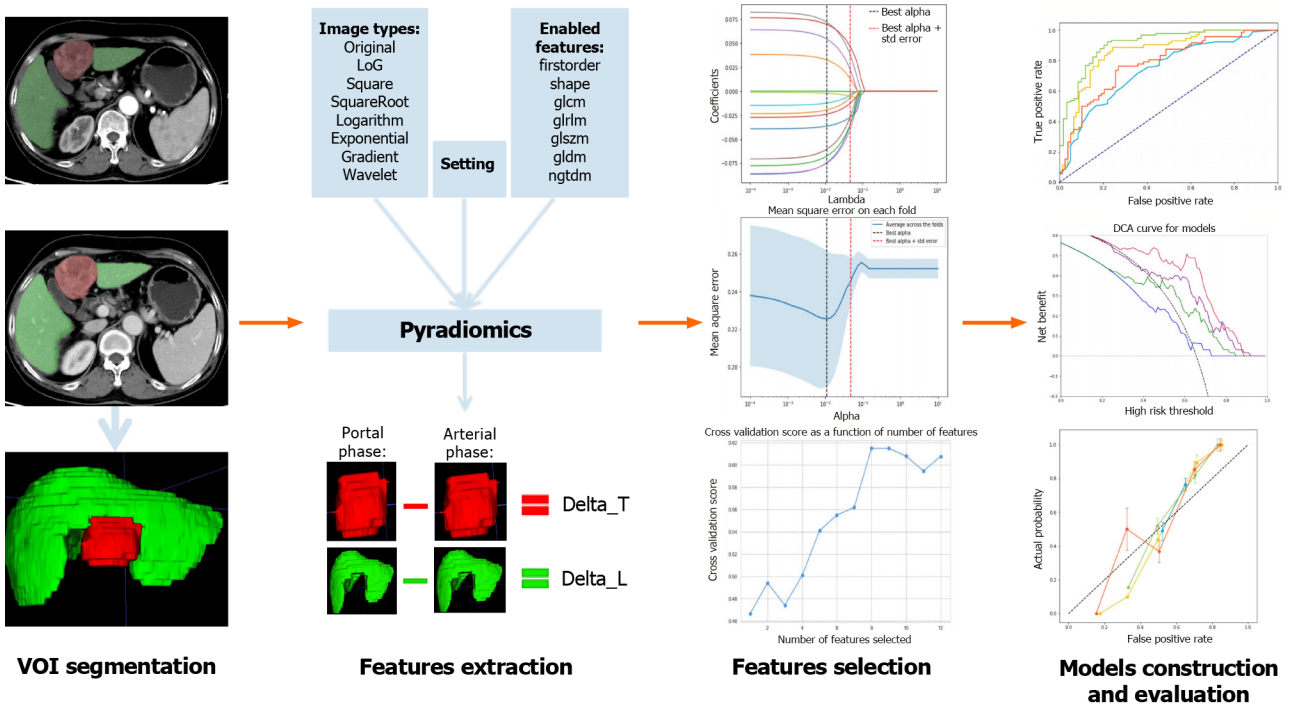

Figure 1 The workflow of radiomics, including volume of interest delineation, feature extraction, feature selection, and model building and evaluation. VOI: Volume of interest.

over time. This is particularly evident in patients with cirrhosis, where the pathophysiological conditions involve microvascular short-circuiting, compression of portal vein branches and hepatic veins by new modules, and disordered arrangement of hepatocyte cords[12]. In patients with cirrhosis, the liver buffer effect is continuously activated, resulting in compensatory arterial blood flow while the portal blood flow is reduced[13]. Delta-radiomics, by integrating the temporal component and radiomics features, provides additional insights into the evolution of feature values. The fixed time intervals of enhanced computed tomography (CT) images obtained during the arterial and portal phases lay the foundation for establishing delta-radiomics. Delta-radiomics features exhibit potential in diagnosing, prognosing, and predicting the therapeutic effects of certain tumours[14,15]. Han *et al*[16] used delta-radiomics features to predict the main pathological response of patients with non-small cell lung cancer to neoadjuvant chemotherapy and immunotherapy. The results indicated that the model formed by delta-radiomics features outperformed the single radiomics model. Based on the delta-radiomics model comprising eight features, Han *et al*[17] achieved an area under the curve (AUC) of 0.805 and 0.857 in the training and validation cohorts, respectively.

Several prior studies have explored the correlation between radiomics and HCC ER after curative-intent hepatectomy [18-22]. It is essential to note that these previous studies focussed solely on extracting radiomics features from tumours and peritumoural tissues. However, this study specifically addresses the background of cirrhosis, where the degree of cirrhosis in the remaining liver parenchyma varies subtly among different patients. Consequently, the analysis of radiomics characteristics encompasses not only the tumour tissue but also the residual liver tissue in this study.

This study aims to develop a machine learning model for predicting ER in HCC patients with cirrhosis. Additionally, the study aimed to assess whether the predicted outcomes of the model could accurately stratify patient overall survival (OS).

MATERIALS AND METHODS

This retrospective study was approved by the Institutional Review Board of our institution (2021-RE-043), and the need for written informed consent was waived.

Clinical information

From January 2014 to June 2020, 563 patients underwent hepatectomy for HCC at the First Affiliated Hospital of the University of Science and Technology of China (Anhui Provincial Hospital). Personal information, clinical information, and imaging features were retrospectively collected. The clinical index data and personal information, encompassing age and sex, for all included participants, were retrieved from the electronic medical records using the hospital system and the corresponding patient hospitalisation number.

HCC diagnosis was confirmed by postoperative pathology. The diagnosis of cirrhosis was based on clinical symptoms, laboratory tests, and imaging examinations, identifying compensated cirrhosis[23]. Curative resection was defined as the complete removal of all detectable tumour nodules by preoperative imaging and intraoperative exploration. This includes

criteria such as negative liver margin pathology, the absence of gross vascular and biliary invasion, no lymph node or extrahepatic distant metastasis, and, for most serum alpha-fetoprotein (AFP) positive patients, normalisation of marker levels within 2 months after surgery, coupled with imaging showing no new tumours. The exclusion criteria were as follows: (1) Non-cirrhotic patients; (2) tumours with extrahepatic metastasis or invasion of major blood vessels; (3) inability to obtain complete enhanced CT images or preoperative enhanced CT within a month; and (4) patients who underwent preoperative treatments such as partial hepatic resection, ablation, transarterial chemoembolisation, and other interventions. A total of 214 eligible patients were included in the study, and they were randomly assigned in a 7:3 ratio to the training cohort (150 patients) and the validation cohort (64 patients). The specific workflow is depicted in Figure 2.

CT equipment

All imaging procedures were performed using the GE Discovery HD 750 multi-row spiral CT scanner. Initially, a routine plain abdominal CT scan was performed on all patients to assess the extent of the lesions. During the scanning process, the abdominal CT parameters, including voltage, current, scanning layer thickness, layer spacing, and pixel matrix size, were set at 120 kV, 200-350 mA, 5 mm, 5 mm, and 512 × 512, respectively. After the non-enhanced CT scan, each patient received a non-ionic iodine contrast injection at a rate of 3.0 mL/s with a dose of 1.5 mL/kg. The arterial phase scan commenced 35 s after the density of the descending aorta reached 95 HU, followed by the initiation of the portal phase scan 35 s after the arterial phase scan.

Imaging information

Digital imaging and communications in medicine-formatted CT images of all patients were retrieved from the hospital picture archiving and communication system. During the assessment, two readers, each possessing extensive experience in liver imaging (Xu ZL and Lu JL), independently recorded nine semantic features: Non-peripheral washout, maximum tumour diameter, tumour capsule, intratumour vascularity, tumour growth pattern, fusion lesions, intratumour necrosis, peritumoural enhancement, and arterial phase hyperenhancement. Simultaneously, the three-dimensional volume algorithm was employed to calculate the tumour volume. In cases where multiple lesions were present in the patient's liver, the tumour with the maximum diameter was selected as the subject for evaluation. To provide a more visual understanding of these imaging semantic features, reference images are included for illustration (Figure 3).

Follow-up

All discharged patients were subjected to regular follow-ups, encompassing monitoring of serum AFP levels, liver function tests, and abdominal ultrasound within the first month after curative liver resection. Subsequent evaluations for HCC recurrence occurred every 3 or 6 months thereafter. In cases where an unexplained elevation in serum AFP levels or abnormal abdominal ultrasound or enhanced ultrasound findings were observed during follow-up, further assessment was performed through dynamic contrast-enhanced CT or magnetic resonance imaging. Recurrence-free survival was defined as the duration from the date of surgery to the first recurrence, metastasis, or the last follow-up. OS was defined as the time from the date of surgery to death from any cause. The study was reviewed on 31 August 2023.

Image segmentation and feature extraction

The volume of interest (VOI) was defined as the entire tumour or the residual liver, excluding peritumoural vessels or bile ducts. Two readers, Xu ZL and Lu JL, blinded to patient outcomes, delineated the patients' VOI layer by layer using ITK-SNAP (v3.6). To ensure the stability of the extracted features, the interobserver reproducibility of the extracted features between the two readers was evaluated. Subsequently, images from 40 cases in the derivation cohort were randomly selected, and the same segmentation procedure was repeated a month later by the same two radiologists to evaluate intraobserver reproducibility. Interobserver reproducibility was evaluated by calculating the intraclass correlation coefficient (ICC).

The Pyradiomics (v3.1.0) software package in Python (v3.8.4) was used for the extraction of radiomics features. To ensure the reproducibility of the extracted features, standardised calculations of radiomics features were used. Within the Pyradiomics package, the following steps were executed: Considering our hospital's machine had a sampling layer thickness of 5 mm, resampling was performed only for the coronal and sagittal positions, with a resampling size of 1 mm × 1 mm × 5 mm. In terms of image types, the original, square, square root, logarithm, exponential, gradient, Laplacian of Gaussian filter, and wavelet filters were applied to the original image.

The extracted features were classified into seven distinct types: (1) Shape; (2) first-order statistics; (3) grey level co-occurrence matrix; (4) grey level run length matrix; (5) grey level size zone matrix; (6) neighbouring grey tone difference matrix; and (7) grey level dependence matrix. After excluding features that could not be analysed, each patient yielded six groups of features, including tumour and residual liver during the arterial and portal phases, along with delta-radiomics (representing the difference between the tumour and residual liver in the portal and arterial phases). This resulted in 1512 features within each group.

Radiomics and clinical-radiologic feature statistics

Given the substantial number of features and their high dimensions, complete inclusion in the model might increase the risk of overfitting. To mitigate this and enhance generalisation, a three-step feature screening method was implemented. First, features with an ICC of < 0.8 were excluded. Subsequently, the selected features underwent Z-score standardisation and were subjected to the least absolute shrinkage and selection operator (LASSO) fitting. To prevent overfitting resulting from an excessive number of features after LASSO, if the number of features after remained > 1, a decision tree classifier was employed as the kernel, and recursive feature elimination was applied to determine the optimal number of features

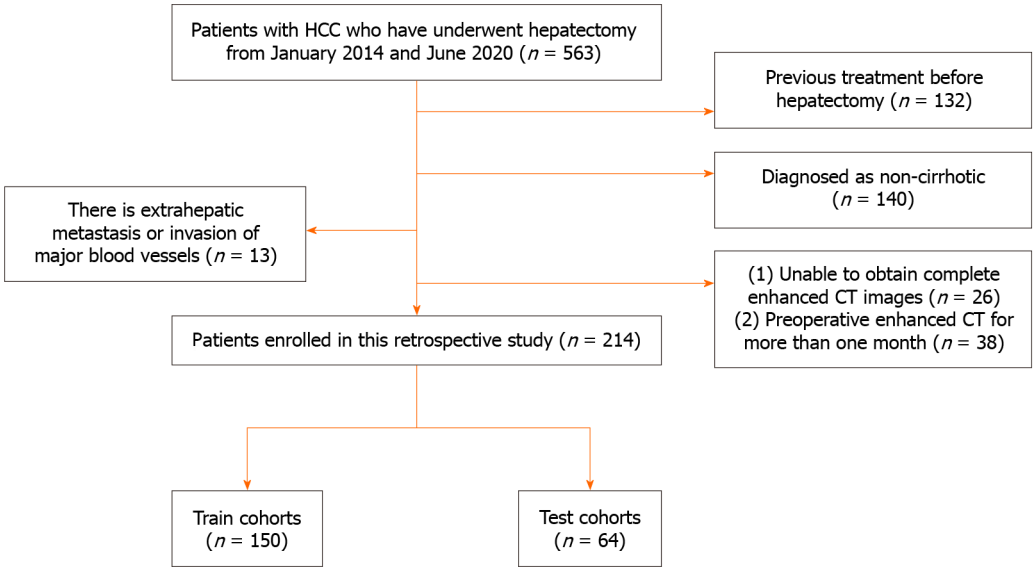

Figure 2 Flowchart illustrating the inclusion and exclusion of patients. HCC: Hepatocellular carcinoma; CT: Computed tomography.

in each group. This screening process was iteratively conducted for each feature group to acquire more representative features. Ultimately, the Rad-score was computed based on the feature-weighted regression coefficient derived from LASSO.

R software (v4.3.0) was employed for analysing clinical-radiologic data. Continuous data were subjected to the *t*-test, and their distribution is presented as the mean ± SD. Categorical data underwent analysis using the chi-square test or Fisher's exact test, and the results are presented as percentages. In the univariate analysis, variables with a P value of < 0.05 were selected and subsequently included in the multivariable logistic regression model for further analysis. All statistical tests were two-sided, with statistical significance set at $P < 0.05$.

Machine learning model building

To minimise discrepancies arising from varying optimal models for different features, this study did not adhere to a fixed model. Instead, the best model was chosen based on the data of each feature, followed by a comparison of the final results. The training cohort was used to identify optimal parameters and develop the prediction model, while the validation cohort was employed to assess prediction performance. The radiomics model was constructed using the scikit-learn (v1.0.2) package in Python, including Support Vector Machine (SVM), Random Forest, K-Nearest Neighbour (KNN), Light Gradient-boosting Machine, and eXtreme Gradient Boosting. A five-fold cross-validation was performed on the training cohort, and the average AUC was calculated. To prevent overfitting and underfitting, models were excluded if their average AUC in the training cohort exceeded or fell below 10% of that in the validation cohort. Subsequently, the AUC of the validation cohort was calculated to evaluate model performance, and the best-selected model was used for the subsequent comparison.

RESULTS

Patient baseline characteristics

This study included 214 patients, among which 114 experienced ER. For model development, 150 patients were randomly assigned to the training cohort. The baseline values of clinical-radiologic characteristics for the training and validation cohorts are presented in Table 1, and no significant differences were observed between the characteristics of the two cohorts ($P > 0.05$). The results of the univariate and multivariate analyses are presented in Table 2, revealing that the final three clinical-radiologic features, namely, gamma-glutamyl transferase (GGT), tumour capsule, and peritumoural enhancement, were included in the subsequent analysis.

Feature selection

To evaluate the characteristics of ER of HCC with cirrhosis, each patient's six feature groups included the following: Arterial phase tumours (A1), arterial phase liver (A2), portal phase tumour (P1), portal phase liver (P2), the characteristic difference between portal and arterial phase tumour (delta-T), and the difference between portal and arterial liver (delta-L). After the feature selecting step, two features were obtained in A1, one feature in A2, three features in P1, three features in P2, and three delta-radiomics (comprising two features in delta-T and one feature in delta-L). In total, 12 features were extracted from the six groups.

Table 1 The clinical-radiologic characteristics of primary cohort, *n* (%)

Variable	Training cohort (*n* = 150)	Validation cohort (*n* = 64)	*P* value
Tumor-volume (cm^3), mean ± SD	249 ± 381	318 ± 361	0.219
Age (yr), mean ± SD	57.3 ± 10.1	54.5 ± 11.7	0.079
Rad-score	0.4 ± 0.2	0.52 ± 0.16	0.121
BMI			0.691
0, < 18.5	7 (4.67)	3 (4.69)	
1, 18.5-25	109 (72.7)	43 (67.2)	
2, ≥ 25	34 (22.7)	18 (28.1)	
AFP (ng/mL)			0.382
0, ≤ 400	91 (60.7)	34 (53.1)	
1, > 400	59 (39.3)	30 (46.9)	
sex			0.839
0, male	128 (85.3)	56 (87.5)	
1, female	22 (14.7)	8 (12.5)	
Hepatitis (HBV/HCV)			0.407
0, absent	22 (14.7)	6 (34.4)	
1, present	128 (85.3)	58 (90.6)	
N (× 10^9/L)			0.275
0, < 1.8	127 (84.7)	54 (84.4)	
1, 1.8-6.3	18 (12.0)	10 (15.6)	
2, > 6.3	5 (3.33)	0 (0.00)	
L (× 10^9/L)			0.504
0, ≥ 1.1	119 (79.3)	54 (84.4)	
1, < 1.1	31 (20.7)	10 (15.6)	
PLT (× 10^9/L)			0.703
0, > 100	120 (80.0)	49 (76.6)	
1, ≤ 100	30 (20.0)	15 (23.4)	
ALT (U/L)			0.959
0, ≤ 50	117 (78.0)	49 (76.6)	
1, > 50	33 (22.0)	15 (23.4)	
AST (U/L)			0.854
0, > 40	67 (44.7)	27 (39.1)	
1, ≤ 40	83 (55.3)	39 (60.9)	
GGT (U/L)			0.113
0, ≤ 60	78 (52.0)	25 (43.8)	
1, > 60	72 (48.0)	39 (56.2)	
TB (umol/L)			0.605
0, ≤ 21	110 (73.3)	44 (68.8)	
1, > 21	40 (26.7)	20 (31.2)	
ALB (g/L)			0.720
0, ≤ 40	76 (50.7)	30 (46.9)	
1, > 40	74 (49.3)	34 (53.1)	

NLR			0.697
0, ≤ 2	74 (49.3)	29 (45.3)	
1, > 2	76 (50.7)	35 (54.7)	
PLR			0.528
0, ≥ 95	78 (52.0)	37 (57.8)	
1, < 95	72 (48.0)	27 (42.2)	
HbsAg			1
Negative	18 (12.0)	8 (12.5)	
Positive	132 (88.0)	56 (87.5)	
BCLC			0.554
0, stage0	7 (4.67)	5 (7.81)	
1, stageA	143 (95.3)	59 (92.2)	
CNLC			0.308
0, Ia	74 (49.3)	26 (40.6)	
1, Ib	76 (50.7)	38 (59.4)	
Non-peripheral washout			0.738
0, absent	2 (1.33)	2 (3.12)	
1, present	148 (98.7)	62 (96.9)	
Tumor capsule			0.110
0, ill-defined capsule	58 (38.7)	33 (51.6)	
1, well-defined capsule	92 (61.3)	31 (48.4)	
Intratumor vascularity			0.654
0, absent	24 (16.0)	8 (12.5)	
1, present	126 (84.0)	56 (87.5)	
Tumor growth pattern			0.635
0, intrahepatic growth	56 (37.3)	21 (32.8)	
1, extrahepatic growth	94 (62.7)	43 (67.2)	
Fusion lesions			0.255
0, absent	87 (58.0)	31 (48.4)	
1, present	63 (42.0)	33 (51.6)	
Intratumor necrosis			0.05
0, absent	47 (31.3)	11 (17.2)	
1, present	103 (68.7)	53 (82.8)	
Peritumoral enhancement			1
0, absent	90 (60.0)	39 (60.9)	
1, present	60 (40.0)	25 (39.1)	
Tumor margin			0.76
0, smooth	84 (56.0)	38 (59.4)	
1, non-smooth	66 (44.0)	26 (40.6)	
Arterial phase hyperenhancement			0.738
0, absent	2 (1.33)	2 (3.12)	
1, present	148 (98.7)	62 (96.9)	

BMI: Body mass index; AFP: Alpha fetoprotein; N: Neutrophil; L: Lymphocyte; ALT: Alanine transferase; AST: Aspartate transferase; GGT: Gamma-glutamyl transferase; PLT: Platelets; TB: Total bilirubin; ALB: Albumin; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; BCLC: Barcelona Clinic Liver Cancer; CNLC: China Liver Cancer Staging System.

Table 2 Of 150 patients univariate and multivariate logistic regression analysis

Variable	Univariable			Multivariable		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Tumor-volume	1.00	1.00-1.00	0.022	1.00	1.00-1.00	0.189
Rad-score	238.02	20.57-2754.04	0.001	298.44	12.57-7083.67	< 0.001
AST						
0, > 40						
1, ≤ 40	0.47	0.25-0.91	0.025	0.93	0.38-2.29	0.875
GGT						
0, ≤ 60						
1, > 60	3.99	2.02-7.86	0.001	2.50	1.07-5.86	0.034
CNLC						
0, Ia						
1, Ib	2.26	1.17-4.35	0.015	1.02	0.39-2.63	0.975
Capsule appearance						
0, ill-defined						
1, well-defined capsule	0.44	0.23-0.86	0.017	0.33	0.14-0.77	0.01
Peritumoral enhancement						
0, absent						
1, present	3.21	1.62-6.34	0.001	3.85	1.67-8.88	0.002

AST: Aspartate transferase; GGT: Gamma-glutamyl transferase; CNLC: China Liver Cancer Staging System.

Model comparison

Initially, three models were developed based on the radiomics features. Model 1 comprised A1 and P1, Model 2 included A1, P1, A2, and P2, and Model 3 included A1, P1, A2, P2, and delta-radiomics. All three radiomics feature models employed SVM as the optimal machine learning method. The performance effects among the models are illustrated in Figure 4, with Model 3 achieving an AUC value of 0.756 in the validation cohort. Compared with Models 1 and 2 performance, our subsequent study opted for the radiomics features in Model 3. Furthermore, a combined model was established by integrating clinical-radiologic and radiomics features, which was compared with the clinical-radiologic model and the radiomics model. As shown in Table 3, the SVM emerged as the best model established by radiomics, while the KNN was the optimal model established by the clinical-radiologic and combined models.

Combined model construction and performance evaluation

The combined model, integrating both radiomics and clinical-radiologic features, demonstrated enhanced diagnostic and predictive efficacy, achieving an AUC of 0.844 in the training cohort and an AUC of 0.790 in the validation cohort. In contrast, the AUC for the clinical-radiologic model was 0.763 in the training cohort and 0.701 in the validation cohort. The AUC values for radiomics features were 0.726 and 0.756, respectively (Figure 5A and B). The Delong test, presented in Table 4, revealed the superiority of the combined model compared to the clinical-radiologic model. Both the calibration curve and the decision curve analysis curve illustrated the commendable calibration and clinical applicability of the combined model (Figure 5C-F). The optimal cut-off value, determined as the maximum Youden index (0.53) in the combined model training cohort, was applied to the training and validation cohorts. This facilitated the categorisation of patients into low-risk and high-risk groups, and the stratification results of patients' OS were analysed using the Kaplan-Meier (K-M) curve. The K-M curve shows that the optimal cut-off value successfully stratified patients OS (Figure 6).

Table 3 Comparison of machine learning model performance

	Models	Training cohort			Validation cohort		
		Accuracy	Precision	AUC	Accuracy	Precision	AUC
Cli	SVM	0.720	0.750	0.736	0.688	0.867	0.686
	RF	0.720	0.750	0.768	0.688	0.867	0.680
	KNN	0.693	0.667	0.763	0.672	0.756	0.701
	XGB	0.720	0.750	0.768	0.688	0.867	0.680
	LightGBM	0.720	0.750	0.761	0.688	0.867	0.693
Rad	SVM	0.673	0.695	0.726	0.719	0.853	0.756
	RF	0.753	0.761	0.849	0.672	0.744	0.688
	KNN	0.713	0.716	0.809	0.656	0.778	0.690
	XGBoost	0.860	0.849	0.945	0.594	0.700	0.588
	LightGBM	0.727	0.696	0.820	0.688	0.729	0.629
Con	SVM	0.727	0.754	0.778	0.688	0.867	0.739
	RF	0.727	0.731	0.820	0.797	0.872	0.777
	KNN	0.767	0.747	0.844	0.719	0.816	0.790
	XGB	0.840	0.875	0.924	0.609	0.743	0.656
	LightGBM	0.807	0.787	0.892	0.719	0.800	0.771

Five model performance evaluation results from clinical-radiologic, radiomics andcombined models. Cli: Clinical-radiologic model; Rad: Radiomics model; Con: Combined model.

Table 4 Delong test between models

Model	Model	Train		Validation	
		Z	*P* value	Z	*P* value
Cli	Rad	0.757	0.449	-0.791	0.429
Cli	Con	-2.988	0.003	-2.099	0.036
Rad	Con	-3.253	0.001	-0.713	0.476

Cli: Clinical-radiologic model, Rad: Radiomics model, Con: Combined model.

DISCUSSION

HCC is characterised by considerable heterogeneity, and the status of ER in HCC significantly influences the OS. HCC associated with cirrhosis exhibits a higher recurrence rate compared to HCC arising in an individual with a normal hepatic background, thereby contributing to diminished survival rates[6]. In our study, among the 214 patients, 114 patients experienced ER, constituting 53.3% of the total study population. Consequently, the imperative to develop models capable of predicting HCC ER before surgery is of great importance in clinical practice.

Our study revealed that tumour capsule, peritumoural enhancement, GGT, and Rad-score serve as predictors of ER in HCC within the context of cirrhosis. Employing the KNN algorithm, a combined model was established, establishing an AUC of 0.844 in the training cohort and 0.790 in the validation cohort. The Delong test revealed that the difference between the radiomics and clinical-radiologic models in the validation cohort lacked statistical significance, suggesting that the radiomics model can yield predictive results comparable to those of the clinical-radiologic model. Furthermore, the combined model outperformed the clinical-radiologic model in the training and validation cohorts, indicating the pivotal role of radiomics in predicting HCC within the context of liver cirrhosis (Table 4). For patient stratification, the maximum Youden index of the combined model was used as the optimal cut-off value (0.53), categorising patients into ER high-risk and low-risk groups. Subsequently, K-M curve analysis was employed to stratify patients' OS. The results demonstrated a significant difference in the OS between patients in ER high-risk and low-risk groups.

Previous studies have reported that peritumoural enhancement and tumour capsule are predictive factors for postoperative HCC recurrence[23-25]. This association might stem from the correlation between peritumoural en-

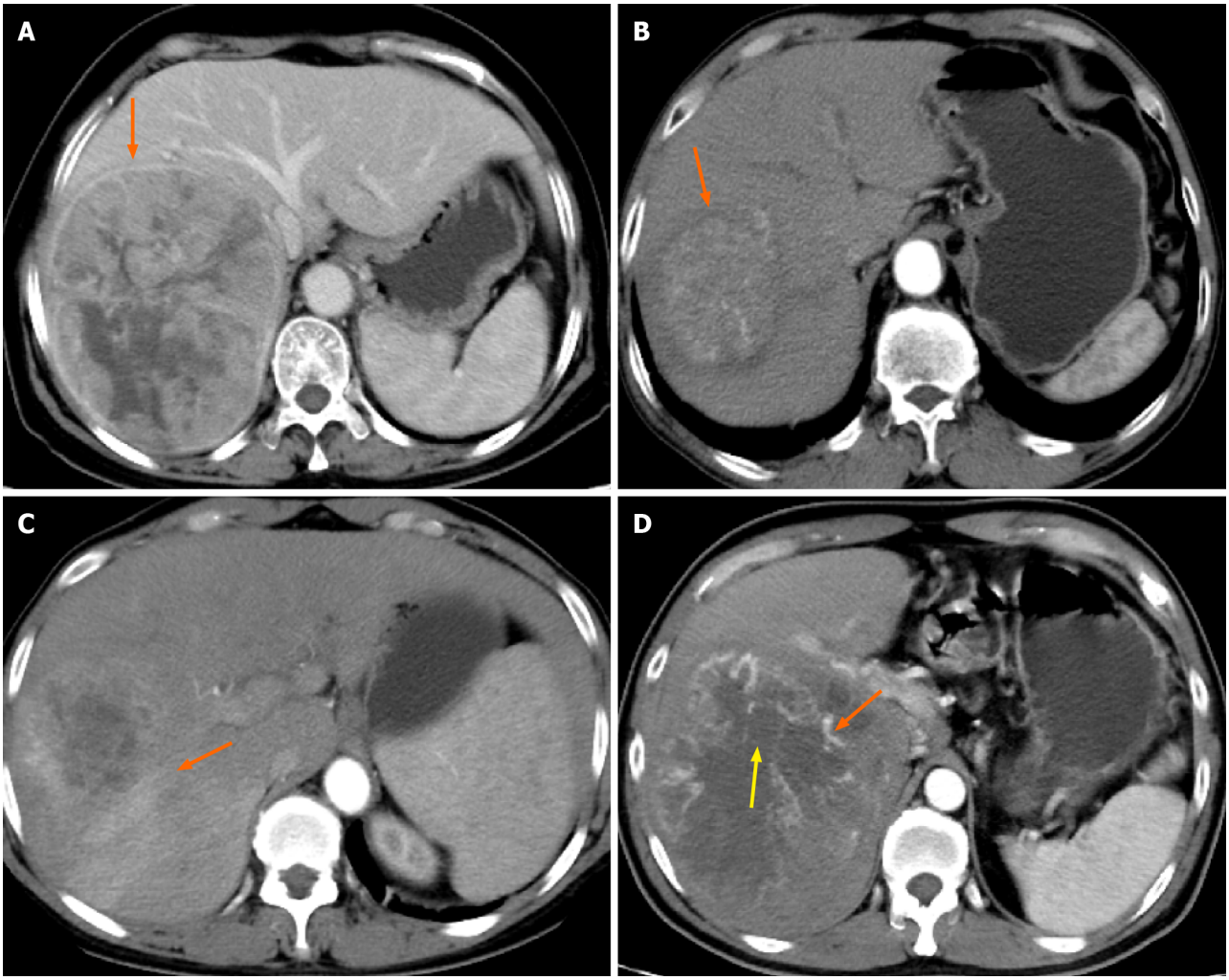

Figure 3 Example of the typical computed tomography images of the semantic features. A: The tumour capsule is observed in the direction indicated by the arrow, with a ring-like high-density appearance around the tumour in the portal phase; B: Smooth tumour margins are observed in the direction of arrows; C: Peritumoural enhancement is visible in the direction of the arrow, characterised by a patchy, high-density area outside the tumour range in the arterial phase; D: Intratumour necrosis within the tumour is observed in the direction of the red arrow, demonstrating a low-density area in the arterial phase. Additionally, the yellow arrow highlights intratumour vascularity, appearing as a linear high-density area within the arterial phase.

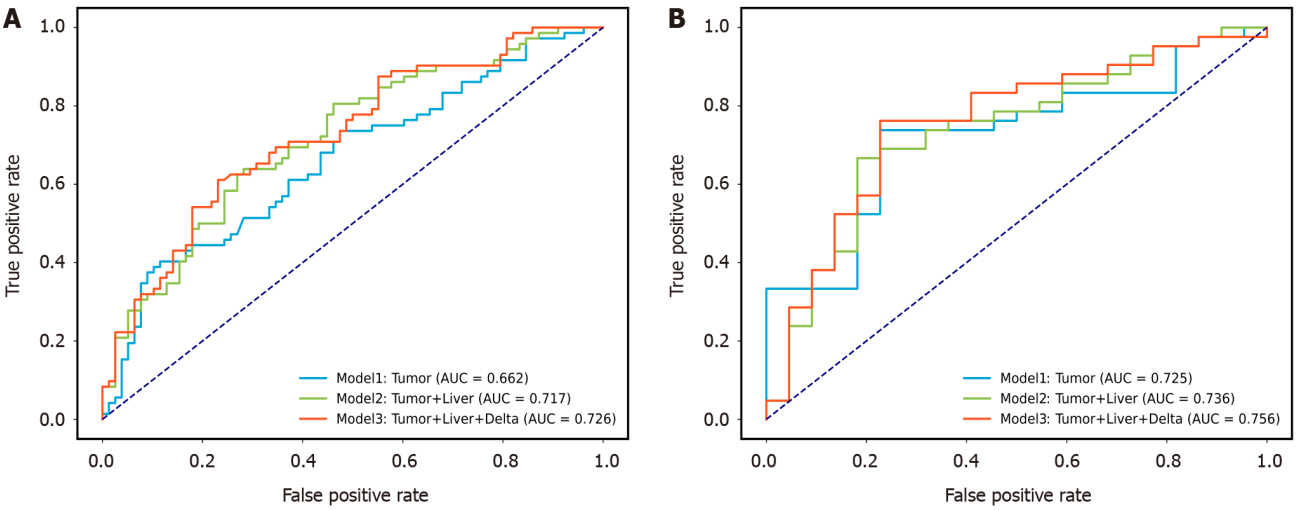

Figure 4 Receiver operating characteristic curve for three radiomics models developed using support vector machine. A: The performance of the three radiomics models in the training cohort; B: The performance of the three models in the validation cohort reveals that the area under the curve of Model 3 was 0.762, and its performance was better than that of Models 1 and 2. AUC: Area under the curve.

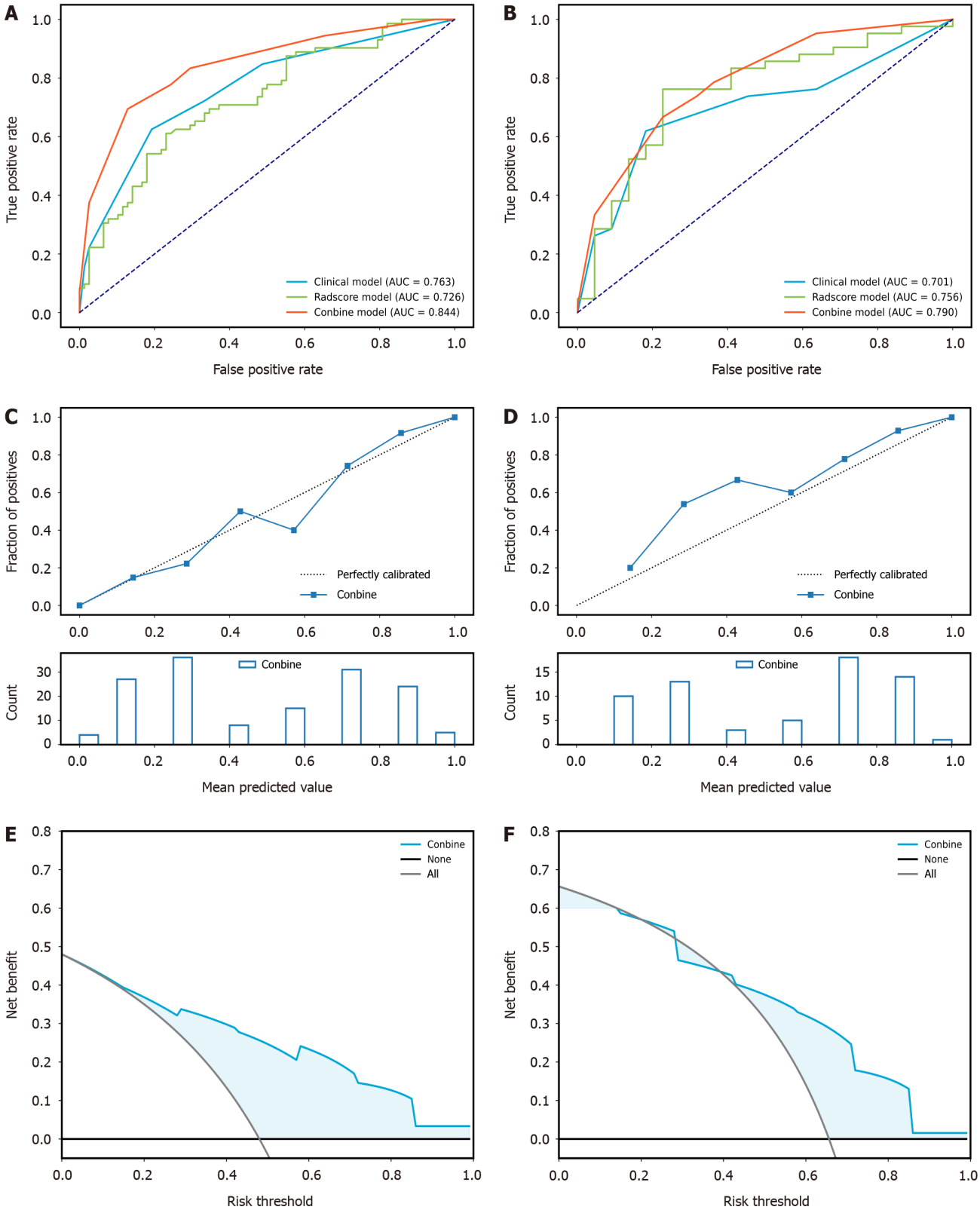

Figure 5 Receiver operating characteristic of the clinical-radiologic model established by K-Nearest Neighbour, radiomics model established by support vector machine, and the combined model established by K-Nearest Neighbour. A: The area under the curve (AUC) values of the clinical-radiologic, radiomics, and combined models in the training cohort were 0.763, 0.726, and 0.844, respectively; B: In the validation cohort, the AUC values of the clinical-radiologic, radiomics, and combined models were 0.701, 0.756, and 0.790, respectively; C: Calibration curve performance of the combined model in the training cohort; D: Combined model calibration curve in the validation cohort; E: Decision curve analysis (DCA) of the combined model in the training cohort; F: DCA of the combined model in the validation cohort. AUC: Area under the curve.

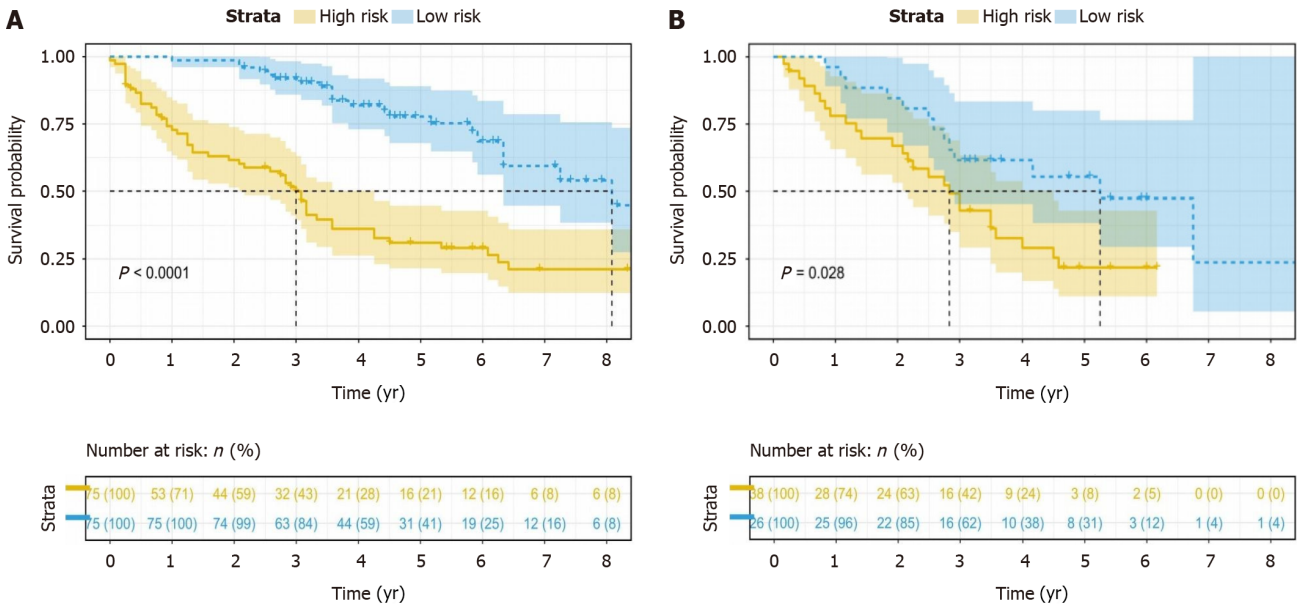

Figure 6 Kaplan-Meier curve analysis of the overall survival of patients stratified based on the best Youden index after hepatectomy. The high-risk and low-risk groups had scores > 0.53 and < 0.53, respectively. A: In the training cohort, the median overall survival (OS) was 3.00 and 8.08 years for the high-risk and low-risk groups, respectively ($P < 0.0001$); B: In the validation cohort, the median OS was 2.83 and 5.25 years for the high-risk and low-risk groups, respectively ($P = 0.028$).

hancement, tumour capsule, and microvascular invasion (MVI) in HCC[26]. Numerous studies have consistently identified MVI as an independent risk factor for ER and unfavourable prognosis after HCC surgery[27,28]. Additionally, GGT has been identified as a predictive factor for adverse outcomes after HCC[29,30]. Our study findings, indicating GGT, tumour capsule, and peritumoural enhancement as independent risk factors for ER of HCC, align with previous studies. However, predicting the ER of HCC solely based on clinical-radiologic factors remains suboptimal (AUC 0.701). Therefore, there is a need for a more precise and convenient method to predict patient outcomes. Radiomics, an emerging imaging analysis approach, uses data mining algorithms to extract features from existing medical images. Subsequently, statistical analysis tools are employed to analyse high-throughput imaging features, providing predictive or prognostic information[10]. Prior to this study, radiomics has been widely used for predicting the diagnosis and prognosis of HCC [31-33]. Notably, delta-radiomics has demonstrated the ability to establish models with high performance. In a study by Xia *et al*[34] a hybrid model combining clinical-radiologic features and delta-radiomics predicted MVI with AUC values of 0.86 and 0.84 in the internal and external validation cohorts, respectively. This model also effectively classified the ER-free rate and OS. Another study by Liu *et al*[35] developed a model based on the AP-TP 5 signal for OS in patients with HCC, achieving AUC values between 0.774-0.837 in the training cohort and 0.754-0.810 in the validation cohort for 1-3 year predictions. Unlike previous studies that primarily discussed the ER of HCC with cirrhosis, previous solely using clinical data[36], our study not only addressed this issue but also incorporated radiomics and compared it with clinical-radiologic features. Ultimately, a combined model with an AUC of 0.790 in the validation cohort was developed.

Past studies often compared clinical-radiologic models, radiomics models, and combined models using the same machine learning methods[37]. However, the optimal models corresponding to different features might vary. To address this issue, our study employed five different machine learning methods and compared the best models corresponding to each feature using specific algorithms, mitigating the bias introduced by different model selections. In this study, three models were compared: one only with tumour features (Model 1), another with tumour and residual liver features (Model 2), and a third with tumour, residual liver, and delta-radiomics features (Model 3). The results revealed the superior performance of Model 3, established with SVM. Simultaneously, this algorithm was used to compare the clinical-radiologic, radiomics, and combined models. The outcomes demonstrated that the combined model, established by KNN exhibited the best performance. This approach allows for a more accurate assessment of the various models' performance and enables the selection of the most suitable model for predicting the prognosis of HCC.

However, it is crucial to acknowledge certain limitations in our study. First, being a retrospective study, the possibility of selection bias could not be eliminated. Second, the study was conducted solely at a single centre, and conducting a multicentre validation study would enhance the reliability of the results. Therefore, it is expected that future studies will involve a multicentre prospective study to validate our findings. Third, the primary aetiology among patients with cirrhosis in our study was hepatitis B and C. Consequently, further verification of the model established by us is warranted in HCC patients with cirrhosis caused by other factors. Fourth, from a machine learning perspective, the sample size in this study is relatively small. Consequently, ongoing efforts will involve the inclusion of new samples to continually train and validate our model. Fifth, in our study, manual delineation was still employed, proving to be time-consuming and resource-intensive. Although attempts were made to employ deep learning networks for training tools to automatically segment the liver and tumour, the results were not sufficiently accurate, primarily due to the limitation of a small sample size. Moving forward, the plan is to expand the sample size to train a deep learning network model capable

of fully automating the segmentation of the patient's tumour and the residual liver.

CONCLUSION

In this study, the combined model for predicting the ER of HCC was constructed using KNN. The AUC values were 0.844 and 0.790 in the training and validation cohorts, respectively. The K-M curves demonstrated that the outcomes predicted by the combined model could correctly stratify patients' OS. The prospective application of this model in clinical practice holds promise for delivering precise, individualised guidance for patient prognosis.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) ranks as the sixth most prevalent tumour and stands as the third leading cause of cancer-related deaths globally. Liver cirrhosis emerges as the primary risk factor for HCC, affecting nearly 90% of patients with HCC to varying degrees. The prognosis for HCC with cirrhosis remains poor, primarily attributable to the elevated recurrence rates.

Research motivation

Individuals with HCC in the background of cirrhosis frequently experience higher recurrence rates compared to patients with HCC in a non-cirrhotic liver. Therefore, the purpose of our study was to establish a model that could predict early recurrence (ER) of HCC within the context of cirrhosis.

Research objectives

To develop a machine learning model to predict the ER of post-hepatectomy HCC in patients with cirrhosis and stratify patients' overall survival (OS) based on the predicted risk of recurrence.

Research methods

In this retrospective study, 214 HCC patients with cirrhosis who underwent curative hepatectomy were examined. Radiomics feature selection employed the least absolute shrinkage and selection operator and recursive feature elimination. Clinical-radiologic features were selected through univariate and multivariate logistic regression analyses. Five machine learning methods were used for model comparison and optimal model selection. The area under the receiver operating characteristic curve (AUC), calibration, and decision curve analysis were used to evaluate the model's performance. The Kaplan-Meier (K-M) curve was used to assess the model's stratification effect on patient OS.

Research results

The optimal performance in predicting ER of HCC within the context of cirrhosis was observed in a model that integrated radiomics features and clinical-radiologic features. This model attained an AUC of 0.844 in the training cohort and 0.790 in the validation cohort. K-M curves demonstrated that the combined model not only allowed for risk stratification but also exhibited significant discrimination in patients' OS.

Research conclusions

The combined model that integrates radiomics and clinical-radiologic characteristics achieved excellent performance in patients with HCC with a background of cirrhosis. K-M curves assessing OS revealed statistically significant differences.

Research perspectives

Given the significant impact of ER on the prognosis of HCC in patients with cirrhosis, accurately predicting such recurrence is paramount. The study aims to investigate the prediction of ER in HCC with cirrhosis using enhanced computed tomography radiomics.

FOOTNOTES

Co-first authors: Gui-Xiang Qian and Zi-Ling Xu.

Author contributions: Qian GX and Jia WD designed the research study; Qian GX, Xu ZL, Li YH, Bo XY, Wei MT and Lu JL collected the data; Xu ZL, Lu JL and Wei MT analyzed the data; all authors wrote the manuscript; Qian GX, Li YH, and Jia WD revised the manuscript; all authors have read and approve the final manuscript. Qian GX and Xu ZL have made equivalent contributions in this article. The reasons are as follows: First, the research covered in this manuscript was a collaborative team effort, with each author dedicating substantial time and effort. Qian GX was responsible for study design, method development, data collection, experimental data analysis, manuscript writing, and subsequent revisions. Meanwhile, Xu ZL played a significant role in data collection, data analysis, and initial manuscript drafting. Second, Xu ZL brings valuable clinical experience to the team. Throughout the research collaboration

with Qian GX, Xu ZL continuously refined the study process, leveraging his accumulated knowledge to identify and rectify potential errors. On the other hand, Qian GX skillfully applied her clinical and machine learning expertise to ensure the study's quality and reliability. Given these reasons, and to accurately reflect the efforts and contributions of each author, I, as the corresponding author, have designated Qian GX and Xu ZL as co-first authors for this study, acknowledging their equal contributions.

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ORIGINAL ARTICLE

Basic Study

Taurine attenuates activation of hepatic stellate cells by inhibiting autophagy and inducing ferroptosis

Sen Li, Qian-Jun Ren, Can-Hao Xie, Yang Cui, Li-Tao Xu, Yi-Dan Wang, Su Li, Xing-Qiu Liang, Bin Wen, Ming-Kun Liang, Xiao-Fang Zhao

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Abstract

BACKGROUND

Liver fibrosis is a compensatory response during the tissue repair process in chronic liver injury, and finally leads to liver cirrhosis or even hepatocellular carcinoma. The pathogenesis of hepatic fibrosis is associated with the progressive accumulation of activated hepatic stellate cells (HSCs), which can transdifferentiate into myofibroblasts to produce an excess of the extracellular matrix (ECM). Myofibroblasts are the main source of the excessive ECM responsible for hepatic fibrosis. Therefore, activated hepatic stellate cells (aHSCs), the principal ECM producing cells in the injured liver, are a promising therapeutic target for the treatment of hepatic fibrosis.

AIM

To explore the effect of taurine on aHSC proliferation and the mechanisms involved.

METHODS

Human HSCs (LX-2) were randomly divided into five groups: Normal control group, platelet-derived growth factor-BB (PDGF-BB) (20 ng/mL) treated group, and low, medium, and high dosage of taurine (10 mmol/L, 50 mmol/L, and 100

mmol/L, respectively) with PDGF-BB (20 ng/mL) treated group. Cell Counting Kit-8 method was performed to evaluate the effect of taurine on the viability of aHSCs. Enzyme-linked immunosorbent assay was used to estimate the effect of taurine on the levels of reactive oxygen species (ROS), malondialdehyde, glutathione, and iron concentration. Transmission electron microscopy was applied to observe the effect of taurine on the autophagosomes and ferroptosis features in aHSCs. Quantitative real-time polymerase chain reaction and Western blot analysis were performed to detect the effect of taurine on the expression of α-SMA, Collagen I, Fibronectin 1, LC3B, ATG5, Beclin 1, PTGS2, SLC7A11, and p62.

RESULTS

Taurine promoted the death of aHSCs and reduced the deposition of the ECM. Treatment with taurine could alleviate autophagy in HSCs to inhibit their activation, by decreasing autophagosome formation, downregulating LC3B and Beclin 1 protein expression, and upregulating p62 protein expression. Meanwhile, treatment with taurine triggered ferroptosis and ferritinophagy to eliminate aHSCs characterized by iron overload, lipid ROS accumulation, glutathione depletion, and lipid peroxidation. Furthermore, bioinformatics analysis demonstrated that taurine had a direct targeting effect on nuclear receptor coactivator 4, exhibiting the best average binding affinity of -20.99 kcal/mol.

CONCLUSION

Taurine exerts therapeutic effects on liver fibrosis *via* mechanisms that involve inhibition of autophagy and trigger of ferroptosis and ferritinophagy in HSCs to eliminate aHSCs.

Key Words: Hepatic stellate cells; Autophagy; Ferroptosis; Molecular docking; Taurine

Core Tip: We have previously demonstrated that treatment of taurine could alleviate liver fibrosis by inhibiting hepatic stellate cell (HSC) activation and inhibiting activated HSC proliferation. Considering the important role of autophagy and ferroptosis in the process of liver fibrosis pathology, we used molecular biology tests and bioinformatic methods to identify the effect of taurine on autophagy and ferroptosis in HSCs *in vitro*. This study demonstrated for the first time that taurine could inhibit autophagy in HSCs to inhibit their activation while triggering ferroptosis and ferritinophagy to eliminate activated HSCs. Taurine has a direct targeting effect on nuclear receptor coactivator 4, exhibiting the best average binding affinity of -23.95 kcal/mol.

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INTRODUCTION

Liver fibrosis is a compensatory response during the tissue repair process in chronic liver injury, finally leading to liver cirrhosis or even hepatocellular carcinoma. It is reported that liver fibrosis has a high incidence rate and mortality in the world[1,2]. Liver fibrosis is a common pathological occurrence and is initiated as a result of chronic liver injury due to alcohol, viral hepatitis, drugs, toxins, nonalcoholic steatohepatitis, autoimmune liver disease, and so on. The pathogenesis of hepatic fibrosis is associated with the progressive accumulation of activated hepatic stellate cells (aHSCs), which can transdifferentiate into myofibroblasts to produce an excess of the extracellular matrix (ECM)[3]. In the normal liver, HSCs are quiescent and contain retinoid (vitamin A) and numerous lipid droplets. However, in response to liver injury, HSCs transform into the highly activated, proliferative, motile, and contractile myofibroblast phenotype by receiving either autocrine or paracrine signaling from injured hepatocytes and immune cells. Myofibroblasts are the main source of the excessive ECM responsible for hepatic fibrosis[4]. Since aHSCs are the principal ECM producing cells in the injured liver, they are a promising therapeutic target for the treatment of hepatic fibrosis.

Autophagy is a conservative way of cell self-degradation, which involves the process of lysosomes engulfing their own cytoplasm or organelles to achieve intracellular nutrition and energy reuse. Autophagy has been implicated in major liver pathologies, such as hepatitis C virus (HCV) infection and hepatocarcinoma. Several studies have shown that autophagy dysfunction can exacerbate liver diseases[5]. For example, a decrease in the number of autophagosomes was found in liver tissue of alcoholic liver disease model rats, while an increased autophagosome number was observed in HCV-infected patients[6]. Besides, in α-1 antitrypsin deficiency, which results in protein aggregates and chronic liver injury, autophagy stimulation reduces the hepatic load of aggregated protein and reverses fibrosis. Furthermore, studies have also showed that autophagy is an important process during HSC activation. Treatment of HSCs with the autophagy

inhibitor bafilomycin A1, hydroxychloroquine, or 3-methyladenine hampered several characteristic features of the activated phenotype, such as proliferation and expression of ACTA2, PDGFR-β, and PROCOL1a1, in both mouse and human-derived HSCs[7,8]. Bafilomycin A1-treated HSCs present a higher number of large lipid droplets when compared with control cells, further suggesting the important role of autophagy in HSC lipid droplet metabolism. The above-mentioned findings also indicate that autophagy may be a therapeutic target for liver fibrosis.

Taurine, a sulfur-containing amino acid, has a wide range of protective activities towards cytotoxicity and oxidative stress produced in hepatocytes or other tissues, especially antioxidation, anti-inflammatory, as well as anti-apoptotic activities[9,10]. In the liver, taurine is an end product of sulfur amino acid catabolism and its biosynthetic ability is reduced in the case of liver diseases. Exogenous supplementation of taurine can prevent liver injury caused by different harmful substances and inhibit ECM deposition in the damaged liver to prevent liver fibrosis[11]. Miyazaki *et al*[12] reported that the anti-fibrogenesis effect of taurine in rats is associated with inhibiting the proliferation of aHSCs. Our previous studies have demonstrated that taurine can inhibit HSC proliferation and promote cell apoptosis significantly *via* mechanisms mainly involving the p38 mitogen-activated protein kinase-c-Jun NH2-terminal kinase-Caspase 9/8/3 pathway[13]. Overall, these results show that taurine can serve as an effective anti-inflammatory agent to prevent liver disease.

To determine the mechanism by which treatment with taurine protects against hepatic fibrosis, the present study was performed to observe the effect of taurine on autophagy and ferroptosis to provide more data on taurine therapy of hepatic fibrosis.

MATERIALS AND METHODS

Materials

Human HSCs (LX-2) were purchased from XiangYa Central Experiment Laboratory, Central South University, Changsha, Hunan Province, China. Dulbecco's minimum essential medium (DMEM) was obtained from Hyclone (Logan, UT, United States). Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, German). Streptomycin sulfate and penicillin were supplied by North China Pharmaceutical, China. Cell Counting Kit-8 (CCK8) was purchased from Beyotime Biotechnology (Shanghai, China). Taurine was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Platelet-derived growth factor-BB (PDGF-BB) was provided by PeproTech (No. L1019).

Culture and treatment of HSCs

HSCs were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin in an incubator with 5% CO_2 at 37 °C. The culture medium was replaced every other day. When the cell density reached approximately 80% confluence, cells were trypsinized and resuspended in DMEM at a density of 1×10^5/mL. For taurine-treated cells, the supernatant was discarded after centrifugation, and the cells were incubated for 48 h in DMEM containing 10, 50, and 100 mmol/L taurine and 20 ng/mL PDGF-BB, while cells in the control group were incubated in DMEM without taurine.

Cell viability assay

Cell viability was evaluated with a CCK8 kit (Beyotime Institute of Biotechnology, C0037) according to the manufacturer's instructions. Briefly, HSCs were plated in a 96-well plates (Sigma, CLS9898) and exposed to various concentrations of the cytotoxic compounds for the indicated times. CCK8 reagent (10 μL) was added to each well and incubated at 37 °C in 5% CO_2 for 4 h, and then the plates were read at 450 nm using the Thermo MK3 Molecular Device (Morrisville, NC, United States).

Estimation of reactive oxygen species level by enzyme-linked immunosorbent assay

Intracellular reactive oxygen species (ROS) level was measured using the oxidation-sensitive fluorescent probe dichlorodihydrofluorescein diacetate (Solarbio, CA1410) according to the manufacturer's instructions.

Estimation of malondialdehyde and glutathione levels by enzyme-linked immunosorbent assay

The relative malondialdehyde (MDA) and glutathione (GSH) concentrations in cell lysates were assessed using enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering institute, A003-1 and A006-2-1) according to the manufacturer's instructions.

Estimation of iron concentration by ELISA

The relative iron concentration in cell lysates was assessed using an iron assay kit (Nanjing Jiancheng Bioengineering institute, A039) according to the manufacturer's instructions.

Observation of autophagosomes and ferroptosis features by transmission electron microscopy

HSC-LX2 cells were seeded onto a 4-well chambered coverglass at a density of 2×10^4 cells/mL (14000 cells/well). Images were acquired using a HIATACHI HT7700 transmission electron microscope.

RNA isolation and real-time polymerase chain reaction

Total RNA was isolated and quantitative polymerase chain reaction (PCR) was performed using the QuantiTect SYBR Green PCR Kit (Thermo, F-415XL) in accordance with the manufacturer's instructions. Beta-actin levels were taken for normalization and fold change was calculated using 2-ddct. The sequence of primers used is showed in Table 1.

Western blot analysis

HSC cells were lysed using a mammalian lysis buffer (Beyotime, P0013B) and immunoblotting was performed according to the manufacturer's guidelines (Bio/Rad, Hercules, CA, United States). Densitometry analysis was performed using the ImageJ software.

Statistical analysis

All the data are expressed as the mean ± SD and were analyzed with GraphPad Prism (GraphPad Software, San Diego, CA, United States). To compare the data of two groups, unpaired Student's *t*-test was used. One-way analysis of variance with the Bonferroni *post hoc* test was used for multiple group comparisons. *P* values were all two-sided and considered statistically significant when they were < 0.05.

RESULTS

Taurine suppresses PDGF-BB-induced HSC proliferation

The effect of taurine on aHSC viability was assessed by the CCK8 assay. There was no significant difference in aHSC viability among the groups at 0 and 24 h. The proliferation of HSCs (LX-2) was promoted by PDGF-BB (20 ng/mL) at 48 and 72 h ($P < 0.01$, Figure 1A). aHSC viability was significantly decreased in cells cultured with different concentrations of taurine for 48 h compared to those cultured without taurine but activated by PDGF-BB (Figure 1A). Therefore, we selected 48 h as the treatment duration and 50 mmol/L as the working concentration in subsequent experiments.

Taurine inhibits expression of α-SMA, Collagen I, and Fibronectin 1

The protein expression of α-SMA, Collagen I, and Fibronectin 1 in the PDGF-BB-induced groups was significantly higher than that in the control group. However, the upregulated expression of α-SMA, Collagen I, and Fibronectin 1 induced by PDGF-BB was significantly inhibited by taurine ($P < 0.01$, Figure 1B and C).

Taurine induces autophagy in HSCs

Western blot analysis showed that taurine significantly inhibited the expression of p62 and increased the expression of LC3B, ATG5, and Beclin 1 (Figure 2A). Transmission electron microscopy showed that typical ferroptosis features were present after taurine intervention: Vacuoles appeared in cells, the edge of nuclear membrane was depressed, and the number of autophagosomes was significantly increased (Figure 2B).

Taurine induces ferroptosis in HSCs

Western blot analysis showed that taurine significantly inhibited the expression of GPX4 protein, but significantly increased the expression of PTGS2 and SLC11A2 proteins (Figure 3A). When ferroptosis occurs, lipid peroxidation is enhanced. We found that taurine significantly increased the level of MDA but significantly decreased the level of GSH. Flow cytometry revealed that taurine significantly increased the levels of ROS (Figure 3B and C). Transmission electron microscopy showed that taurine-treated HSCs showed obvious ferroptotic cell morphological changes, including obvious reduction and shrinkage of mitochondria, disappearance of mitochondrial cristae, and significant mitochondrial shortening (Figure 3D). These results indicate that taurine can induce ferroptosis in HSCs. In addition, taurine significantly increased the deposition of iron ions in HSCs (Figure 3E).

Effect of taurine on ferritinophagy of HSCs

Bioinformatics showed that there is a direct interaction between nuclear receptor coactivator 4 (NCOA4) and ferritin heavy chain 1 (FTH1) (Figure 4), and taurine has a good docking effect with NCOA4 and FTH1, indicating that taurine may have a direct targeting effect on NCOA4 (Figure 4B).

DISCUSSION

It is well known that nearly half of the disease deaths in the developed countries are closely related to chronic fibroproliferative diseases, especially hepatic fibrosis[14-16]. HSC activation is associated with the development of hepatic fibrosis and inhibiting activated HSC (aHSC) proliferation has been identified as an important way for prevention and treatment of hepatic fibrosis[17]. Studies over the past decade have implicated the pivotal role of ferroptosis in the event of HSC activation[18,19]. Although taurine could protect against hepatic fibrosis in rats by inhibiting aHSC proliferation[20,21], there are no in-depth reports focusing on the effect of taurine on HSC ferroptosis in hepatic fibrosis. Elucidation of the mechanisms governing the ferroptosis of aHSCs may provide a therapeutic approach for taurine to control liver fibrosis. In the current study, we initially demonstrated that taurine inhibited the proliferation of aHSCs *in vitro*, as manifested by

Table 1 Sequence of primers used

Gene	Forward	Reverse
LC3B	5′-ACAAGGGTGAGAAGCA-3′	5′-ACCAGCAGGAAGAAGG-3′
ATG5	5′-CGAGATGTGTGGTTTGG-3′	5′-ATTTCAGTGGTGTGCCT-3′
Beclin1	5′-ATACCGACTTGTTCCTT-3′	5′-GTCTTCAATCTTGCCTT-3′
p62	5′-TACCTGCCCGAACTCA-3′	5′-AATCTTCCCCACAAAA-3′
CollagenI	5′-GCCAATGTGGTTCGTG-3′	5′-TGGGCTGAGTGGGGTA-3′
Fibronectin1	5′-CTGGAGGAGACCACATGAGACTG-3′	5′-TCCTTGTGTCCTGATCGTTGCATC-3′
PTGS2	5′-AGGTGTATGTATGAGTGTG-3′	5′-AGTGGGTAAGTATGTAGTG-3′
SLC11A2	5′-GGAGCAGTGGCTGGATT-3′	5′-CGTGGGACCTTGGGATA-3′
GPX4	5′-CCGCTGTGGAAGTGGATG-3′	5′-CGCTGGATTTTCGGGTCT-3′
α-SMA	5′-TGCTCCCAGGGCTGTTTT-3′	5′-TTGCTCTGTGCTTCGTCA-3′
GAPDH	5′-AGAAGGCTGGGGCTCATTTG-3′	5′-AGGGGCCATCCACAGTCTTC-3′

the observation that cellular viability and the expression of ECM decreased significantly, respectively. Thus, it is conceivable that taurine can inhibit PDGF-BB-induced activation of HSCs and promote their death to play a role in anti-fibrosis.

In recent years, many studies have found that drugs can improve the pathological damage of liver fibrosis by regulating ferroptosis in HSCs. Recently, Zheng *et al*[2] indicated that curcumol induced ferroptosis in HSCs by promoting autophagy and mediating the degradation of NCOA4 and FTH1 complexes to release iron ions[2]. Moreover, Kuo *et al* reported that chrysophanol can impair HBx-induced activation of HSCs *via* endoplasmic reticulum stress and ferroptosis-dependent or *GPX4*-independent pathways[23]. Furthermore, Zhang *et al*[22] indicated that dihydroartemisinin could trigger ferroptosis to eliminate aHSCs by regulating iron overload, lipid ROS accumulation, glutathione depletion, and lipid peroxidation[22]. In our study, we showed that taurine could inhibit aHSC proliferation *in vitro*. The induction of ferroptosis is required for taurine to inhibit HSC proliferation. Our findings together with previous reports indicated that taurine triggered ferroptotic events including iron overload, lipid ROS generation, GSH depletion, and lipid peroxidation product (MDA) accumulation. Furthermore, results also showed that taurine can upregulate the expression of PTGS2 and SLC11A2 proteins and downregulate the expression of GPX4 protein. Our above results are consistent with those reported in previous studies[2,15,22,24]. According to our results, the expression of PTGS2 in PDGF-BB-induced aHSCs was significantly upregulated. PTGS2, also known as cyclooxygenase-2, is the key enzyme in prostaglandin biosynthesis, and acts as both a peroxidase and a dioxygenase[25,26]. It is showed that PTGS2 is involved in the process of ferroptosis because it was significantly upregulated after treatment with Rsl3 and erastin in mice[15,27]. As a matter of fact, PTGS2 was found to be significantly elevated in cells undergoing ferroptosis[28]. Although the exact role of *PTGS2* in the ferroptotic cell death cascade remains to be elucidated, targeting on PTGS2, as being associated with ferroptosis, is an effective way to promote cell death. Additionally, existing studies have elucidated that SLC11A2 activation or upregulation increases ferroptosis in hypoxia/reoxygenation treated myocardial cells[29], and SLC11A2 knockdown reduces iron deposition and lipid peroxidation and therefore alleviates ferroptosis in rats after subarachnoid hemorrhage[28]. Otherwise, GPX4 is equipped with GSH to prevent cells from ferroptosis by reducing oxidized phospholipids, ROS production, and iron uptake. Here, we found that the expression of SLC11A2 was significantly upregulated in PDGF-BB-induced aHSCs after taurine treatment, while the expression of GPX4 was significantly downregulated. Altogether, these data indicate that taurine stimulates ferroptosis in aHSCs by increasing PTGS2 and SLC11A2 expression and decreasing *GPX4* expression to promote cell death. Although much more research is needed to uncover the molecular mechanism of ferroptosis in aHSCs, taurine treatment by inducing ferroptosis has become a potential strategy for eliminating aHSCs.

Autophagy regulates the development of liver injury and fibrosis by affecting the secretion of many cytokines and signal pathways in the liver. The formation of essential autophagy consists of four molecular subunits, which included ATG6/Beclin 1, LC3, ATG9/VMP1, and ULK1 complex[29]. Beclin 1 is a key protein involved in autophagy, and it is also one of the earliest autophagy proteins found in mammals. It plays a key role in the regulation of autophagy, and its up-regulation can stimulate the occurrence of autophagy[30,31]. p62 is a common autophagy protein, its expression is negatively correlated with autophagy level, and it is an important bridge between LC3 and ubiquitin substrate to be degraded. When autophagy is activated, the protein polymer formed by p62 can be degraded by autophagosomes. p62 binds to autophagy membrane protein LC3, thus transporting the protein polymer containing p62 to autophagosomes [32]. Interestingly, we observed that the number of autophagosomes increased in PDGF-BB-induced aHSCs, but it was decreased after treatment with taurine. Meanwhile, our data showed that taurine downregulated the expression of LC3B and Beclin 1 proteins, and upregulated the expression of p62 protein. Thus, it is suggested that taurine can inhibit HSC activation effectively by inhibiting autophagy. Thoen *et al*[33] reported that increased autophagic flux was observed during HSC activation and autophagy can induce HSC activation[33]. In previous studies, several pieces of research have demonstrated that autophagy promotes digestion of lipid droplets in quiescent HSCs, thereby facilitating HSC activation

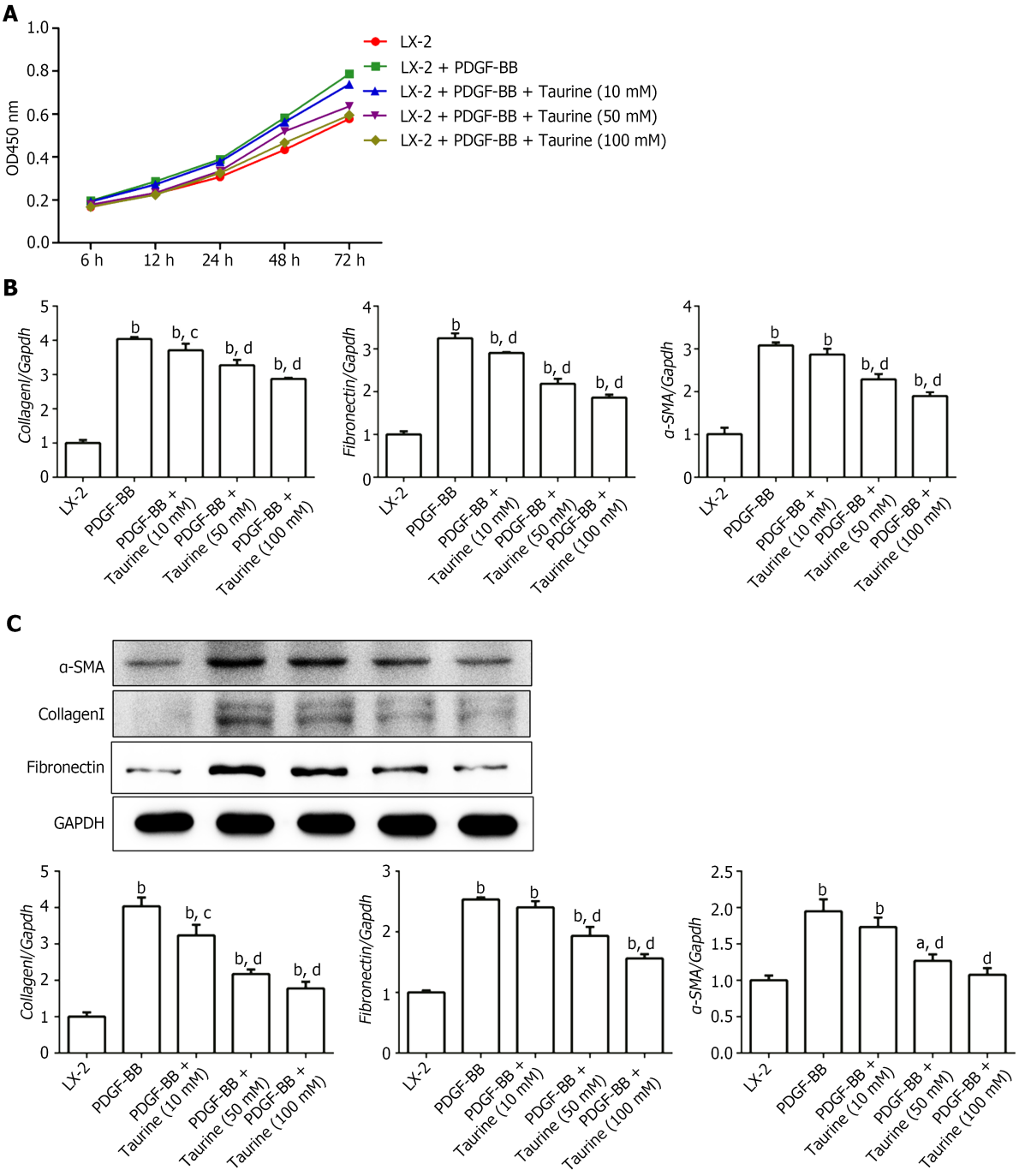


Figure 1 Effects of taurine on extracellular matrix. A: Detection of effect of taurine on the viability of hepatic stellate cells by Cell Counting Kit-8 method; B: Detection of the effect of taurine on mRNA expression of fibronectin 1, collagen I, and α-SMA by reverse transcription-polymerase chain reaction; C: Detection of effect of taurine on protein expression of fibronectin 1, collagen I, and α-SMA by Western blot. Data are expressed as the mean ± SD. $^{a}P < 0.05$ *vs* control, $^{b}P < 0.01$ *vs* control, $^{c}P < 0.05$ *vs* platelet-derived growth factor-BB (PDGF-BB), $^{d}P < 0.01$ *vs* PDGF-BB. PDGF-BB: Platelet-derived growth factor-BB.

and promoting liver fibrosis. Besides, autophagy is regarded as a cytoprotective and anti-fibrotic mechanism in most liver cell types and is crucial for metabolic homeostasis of hepatocytes[34].

Several lines of evidence have indicated a vital relationship between ferroptosis and autophagy[27,35]. Autophagy is identified as an upstream mechanism in the induction of ferroptosis by regulating cellular iron homeostasis and cellular ROS generation[36]. Based on the relationship between autophagy and ferroptosis, Mancias *et al*[37] proposed a new term named ferritinophagy in 2014[37]. Ferritinophagy was regarded as a form of cell-selective autophagy mediated by NCOA4, and it is involved in iron metabolism related pathophysiologic process[16,37]. We did not carry out the experimental verification of NCOA4 knockout and overexpression. Nevertheless, bioinformatics revealed that taurine has a good docking effect with NCOA4. NCOA4 is a key target for regulating the process of ferritin phagocytosis. Studies have showed that NCOA4 depletion inhibits the delivery of ferritin to the lysosome, and NCOA4-mediated ferritinophagy modulates susceptibility to ferroptosis[38]. Besides, Cao *et al*[39] reported that inhibiting autophagy would upregulate the expression of NCOA4 and promote degradation of FTH1, finally promoting ferroptosis in hepatocytes[39]. It was further revealed that NCOA4 is a promising target for anti-hepatic fibrosis. Therefore, many investigators are looking for

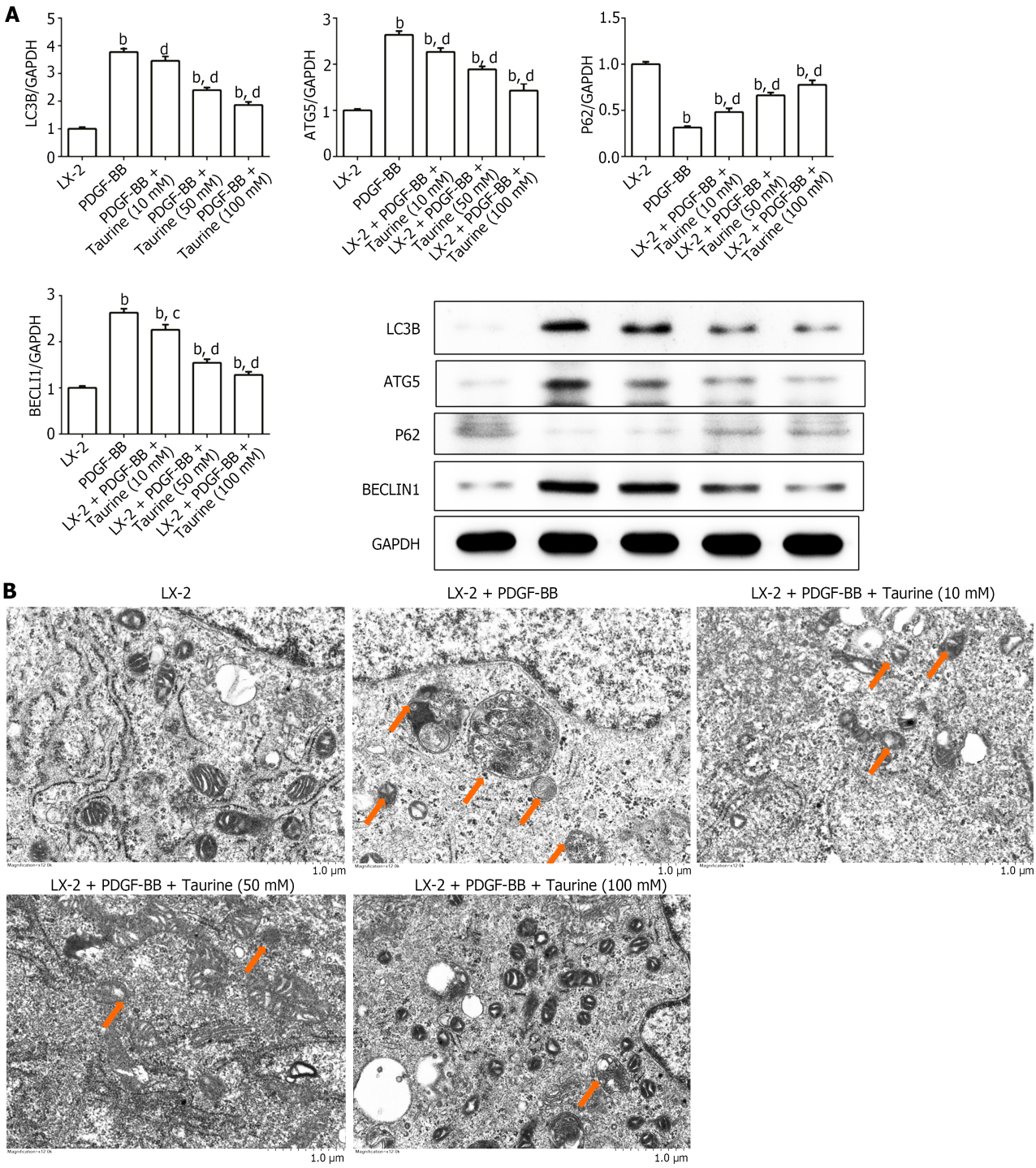
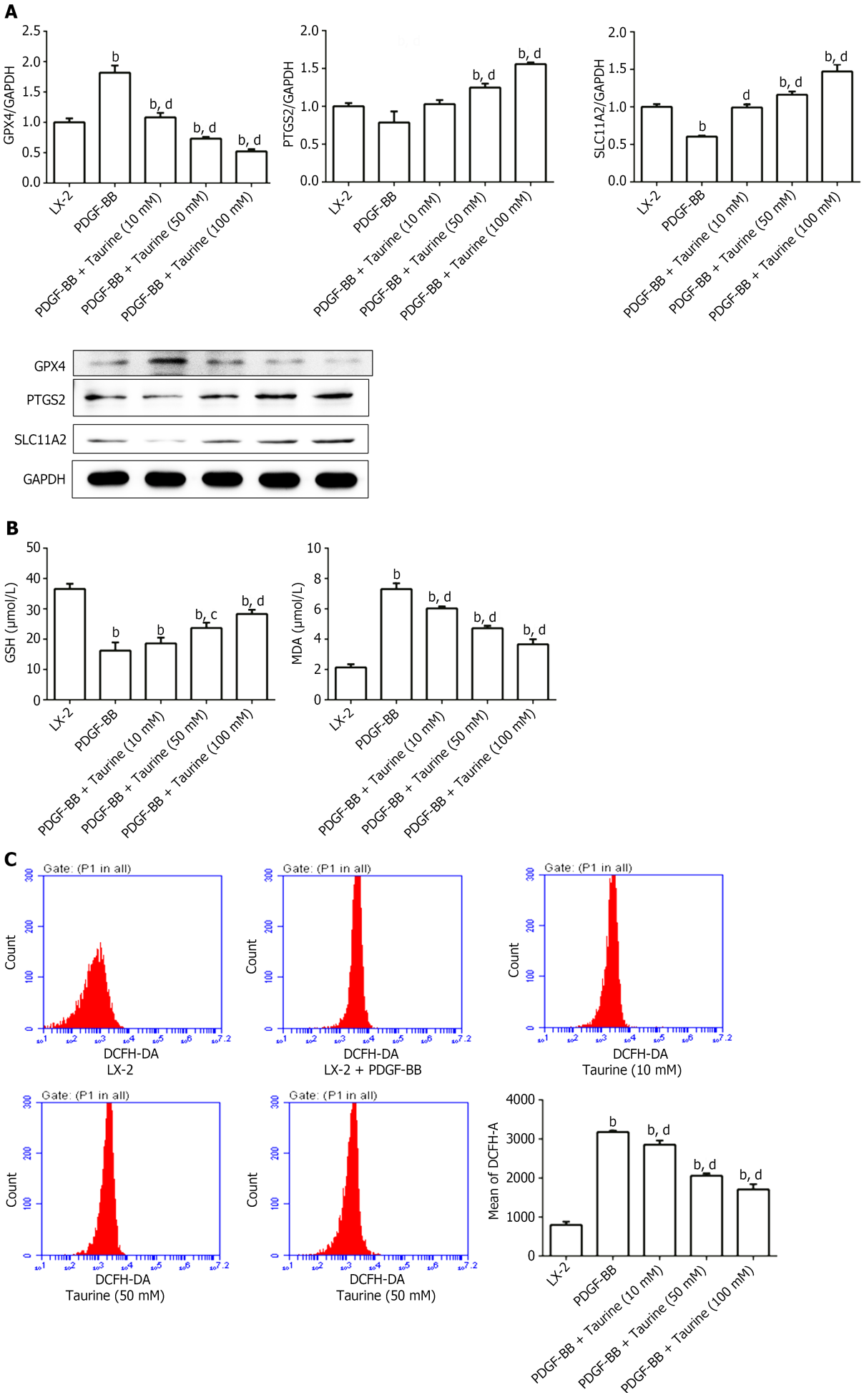


Figure 2 Taurine inhibits autophagy in hepatic stellate cells. A: Effect of taurine on expression of autophagy-related molecules; B: Taurine decreases the number of autophagosomes. Yellow arrows indicate monolayer or bilayer autophagosomes. Bar = 1 μm. Data are expressed as the mean ± SD. $^{a}P < 0.05$ *vs* control, $^{b}P < 0.01$ *vs* control, $^{c}P < 0.05$ *vs* platelet-derived growth factor-BB (PDGF-BB), $^{d}P < 0.01$ *vs* PDGF-BB. PDGF-BB: Platelet-derived growth factor-BB.

strategies to alter the expression of NCOA4 or to regulate HSC ferritinophagy to alleviate liver fibrosis. For example, Zheng *et al*[2] reported that curcumol inhibited the activation of HSCs by increasing the expression of NCOA4 to mediate the migration of FTH1 for degradation in autophagolysosomes[2]. Ma *et al*[16] found that Schisandrin B could ameliorate hepatic fibrosis by inducing NCOA4-mediated ferritinophagy to promote aHSC senescence[16]. Xiu *et al*[29] showed that caryophyllene oxide regulated NCOA4, LC3B, and FTH1 to promote ferritinophagy[29]. Our data provide further evidence for the notion that taurine inhibits HSC activation by regulating the expression of NCOA4 and mediating ferritinophagy.

Altogether, our data demonstrate that the anti-fibrosis mechanisms of taurine include inhibition of autophagy to inhibit HSC activation, and the induction of ferritinophagy and ferroptosis to promote aHSC death (Figure 5).


A
GPX4/GAPDH
PTGS2/GAPDH
SLC11A2/GAPDH
LX-2
PDGF-BB
PDGF-BB + Taurine (10 mM)
PDGF-BB + Taurine (50 mM)
PDGF-BB + Taurine (100 mM)
GPX4
PTGS2
SLC11A2
GAPDH
B
GSH (μmol/L)
MDA (μmol/L)
C
Gate: (P1 in all)
Count
DCFH-DA
LX-2
LX-2 + PDGF-BB
Taurine (10 mM)
Taurine (50 mM)
Mean of DCFH-A

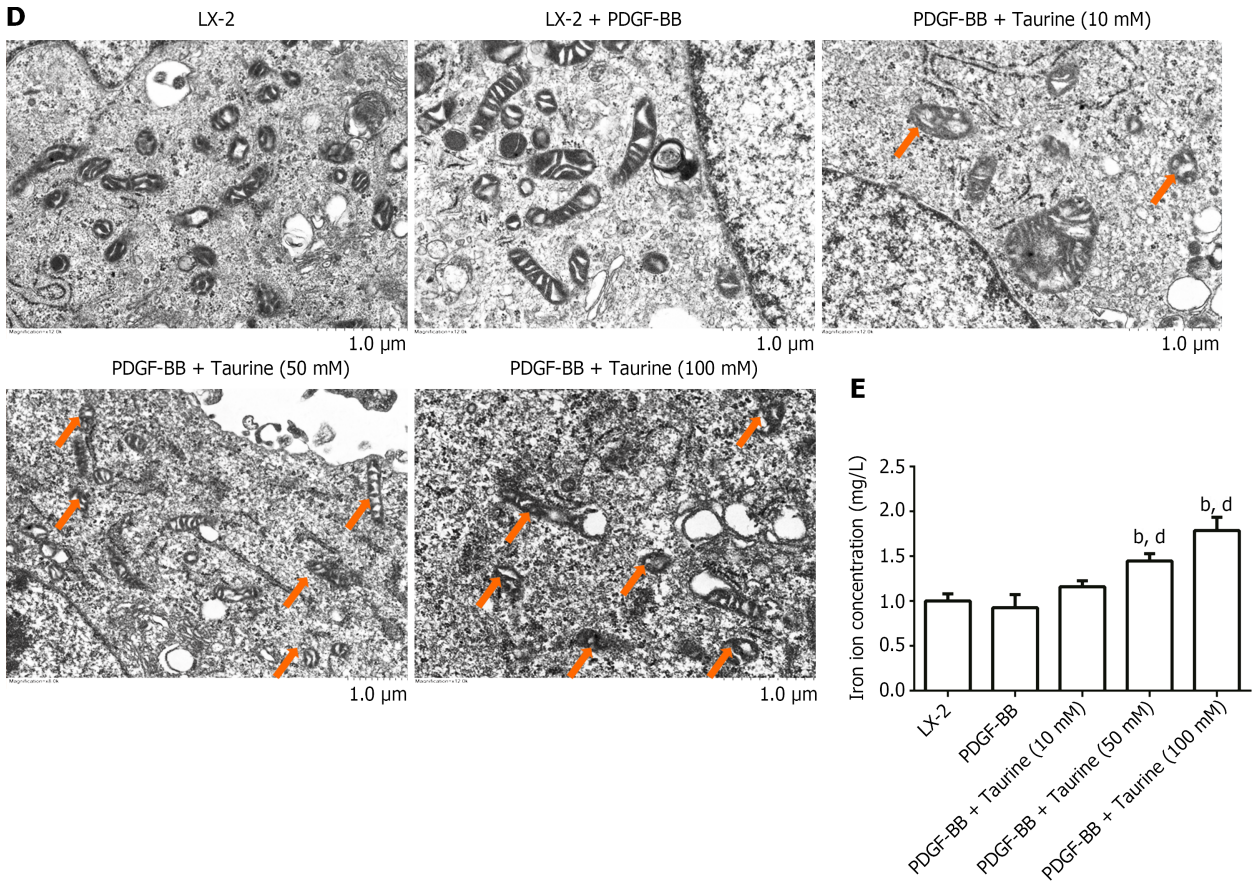


Figure 3 Taurine induces ferroptosis in hepatic stellate cells. A: Effect of taurine on expression of ferroptosis-related molecules; B: Effect of taurine on levels of glutathione and malondialdehyde; C: Effect of taurine on reactive oxygen species; D: Effect of taurine on mitochondrial structure of hepatic stellate cells. Red arrows indicate mitochondria. Bar = 1 μm; E: Taurine induces iron deposition in hepatic stellate cell. Data are expressed as the mean ± SD. $^{a}P < 0.05$ *vs* control, $^{b}P < 0.01$ *vs* control, $^{c}P < 0.05$ *vs* platelet-derived growth factor-BB (PDGF-BB), $^{d}P < 0.01$ *vs* PDGF-BB. PDGF-BB: Platelet-derived growth factor-BB; GSH: Glutathione; MDA: Malondialdehyde.

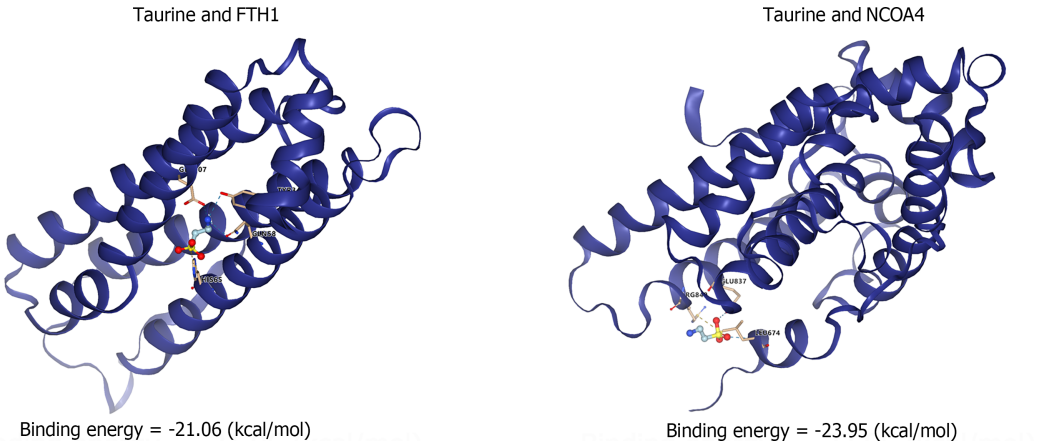


Figure 4 Molecular docking results of taurine and ferritin heavy chain 1 and nuclear receptor coactivator 4, respectively. FTH1: Ferritin heavy chain 1; NCOA4: Nuclear receptor coactivator 4.

CONCLUSION

Taurine inhibits autophagy of HSCs and promotes their ferroptosis and ferritinophagy, thus inhibiting the activation of HSCs to alleviate hepatic fibrosis.

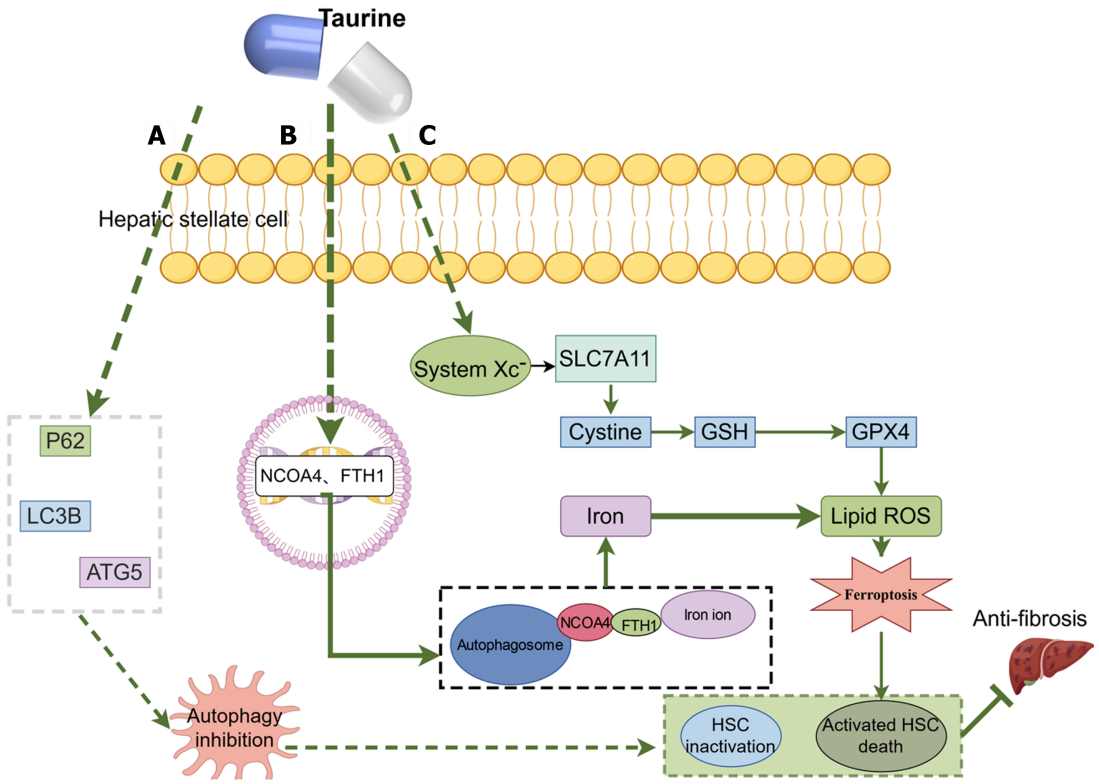


Figure 5 Mechanisms associated with taurine-mediated death and inactivation of hepatic stellate cells in liver fibrosis. A: Inhibition of autophagy; B: Activation of ferritinophagy; C: Induction of ferroptosis. FTH1: Ferritin heavy chain 1; NCOA4: Nuclear receptor coactivator 4; ROS: Reactive oxygen species; HSC: Hepatic stellate cell; GSH: Glutathione.

FOOTNOTES

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Author contributions: Li S drafted the manuscript and conducted the experiments of Western blotting, RT-PCR, and enzyme-linked immunosorbent assay; Ren QJ completed the cell culture and treatment, transmission electron microscopy observation, and visualization of this study; Xie CH, Cui Y, Xu LT, and Wang YD assisted with methodology and validation of this study; Li S and Wen B participated in the supervision of this manuscript; Liang XQ and Wen B contributed to the project administration and funding acquisition; Zhao XF supervised the experiments, corrected the data, revised the manuscript, and provided feedback on the whole manuscript text; Liang MK was responsible for collectively designing, performing, analyzing, and completing the study. All authors contributed to the article and approved the submitted version.

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ORIGINAL ARTICLE

Basic Study

OSW-1 triggers necroptosis in colorectal cancer cells through the RIPK1/RIPK3/MLKL signaling pathway facilitated by the RIPK1-p62/SQSTM1 complex

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Abstract

BACKGROUND

Necroptosis has emerged as a novel molecular pathway that can be targeted by chemotherapy agents in the treatment of cancer. OSW-1, which is derived from the bulbs of *Ornithogalum saundersiae* Baker, exerts a wide range of pharmacological effects.

AIM

To explore whether OSW-1 can induce necroptosis in colorectal cancer (CRC) cells, thereby expanding its range of clinical applications.

METHODS

We performed a sequence of functional experiments, including Cell Counting Kit-8 assays and flow cytometry analysis, to assess the inhibitory effect of OSW-1 on CRC cells. We utilized quantitative proteomics, employing tandem mass tag labeling combined with liquid chromatography-tandem mass spectrometry, to analyze changes in protein expression. Subsequent bioinformatic analysis was conducted to elucidate the biological processes associated with the identified proteins. Transmission electron microscopy (TEM) and immunofluorescence studies were also performed to examine the effects of OSW-1 on necroptosis. Finally, western

blotting, siRNA experiments, and immunoprecipitation were employed to evaluate protein interactions within CRC cells.

RESULTS

The results revealed that OSW-1 exerted a strong inhibitory effect on CRC cells, and this effect was accompanied by a necroptosis-like morphology that was observable *via* TEM. OSW-1 was shown to trigger necroptosis *via* activation of the RIPK1/RIPK3/MLKL pathway. Furthermore, the accumulation of p62/SQSTM1 was shown to mediate OSW-1-induced necroptosis through its interaction with RIPK1.

CONCLUSION

We propose that OSW-1 can induce necroptosis through the RIPK1/RIPK3/MLKL signaling pathway, and that this effect is mediated by the RIPK1-p62/SQSTM1 complex, in CRC cells. These results provide a theoretical foundation for the use of OSW-1 in the clinical treatment of CRC.

Key Words: OSW-1; Necroptosis; RIPK1; P62/SQSTM1; Colorectal cancer

Core Tip: Colorectal cancer (CRC) is a significant health concern worldwide, and it has severe impacts on human lives. Identifying effective drugs for CRC treatment is very important. OSW-1, which is derived from the bulbs of *Ornithogalum saundersiae*, exhibits potent antitumor properties. This study confirmed the inhibitory effect of OSW-1 on CRC through both *in vitro* and *in vivo* experiments. Furthermore, tandem mass tag proteomic analysis was employed to predict differentially expressed proteins and potential underlying mechanisms. Our findings suggest that OSW-1 induces necroptosis *via* the RIPK1/RIPK3/MLKL signaling pathway, and this effect is mediated by the RIPK1-p62/SQSTM1 complex. These results provide a theoretical foundation for the use of OSW-1 in the clinical treatment of CRC.

Citation: Wang N, Li CY, Yao TF, Kang XD, Guo HS. OSW-1 triggers necroptosis in colorectal cancer cells through the RIPK1/RIPK3/MLKL signaling pathway facilitated by the RIPK1-p62/SQSTM1 complex. *World J Gastroenterol* 2024; 30(15): 2155-2174

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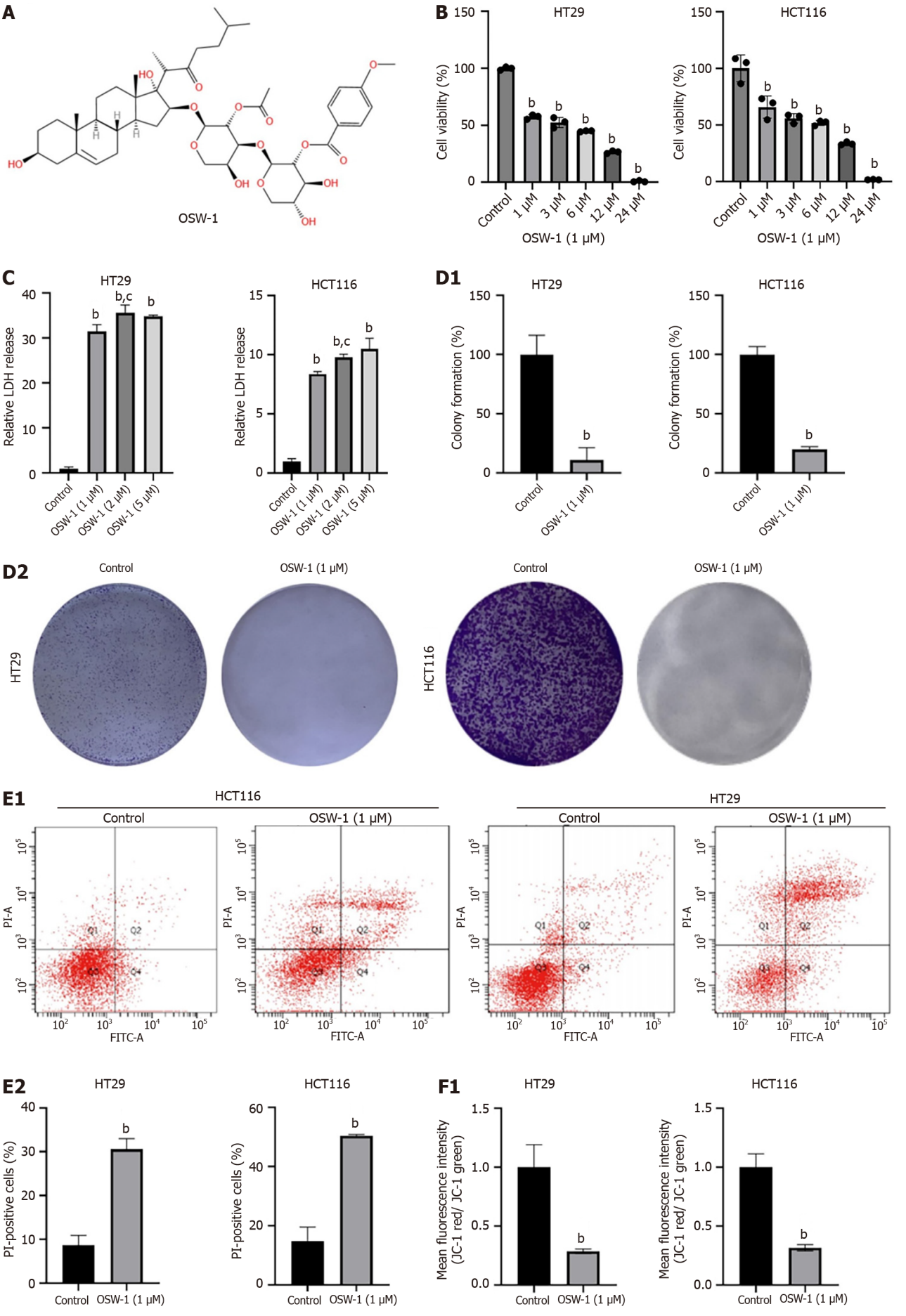
INTRODUCTION

Colorectal cancer (CRC) is the most common type of gastrointestinal tumor, ranking third in incidence among gastrointestinal tumors and posing a significant threat to human health[1]. Globally, CRC was responsible for approximately 9.7% of cancer cases and 8.5% of cancer-related deaths in 2012[2]. While chemotherapy drugs, such as oxaliplatin, irinotecan, and fluorouracil, have demonstrated effectiveness, a growing body of evidence indicates that many treatments are not satisfactorily effect in treating CRC due to issues related to toxicity and drug resistance[3-5].

Ornithogalum saundersiae Baker, which belongs to the Asparagaceae family, is a perennial plant that is native to South Africa and was introduced to China as an ornamental plant. OSW-1 [3β,16β,17α-trihydroxycholest-5-en-22-one-16-O-(2-O-4-methoxybenzoyl-β-D-xylopyranosyl)-(1→3)-(2-O-acetyl-α-L-arabinopyranoside)] (Figure 1A), which is extracted from the bulbs of *Ornithogalum saundersiae*, has been suggested to exert selective toxic effects against cancer cells. The anticancer properties of OSW-1 have been confirmed in diverse types of cancer, including triple-negative breast cancer, hepatocellular carcinomas, and lung cancer[6-9].

The application of quantitative proteomics to investigations of OSW-1-treated CRC cells provides a valuable approach for revealing clinically significant underlying mechanisms. Programmed cell death (PCD) includes various processes, such as necroptosis, apoptosis, pyroptosis, ferroptosis, and autophagy, and it has been shown to be a critical regulator of tissue development and to have significant implications for clinical outcomes. While much research has explored the mechanisms underlying apoptosis in cancer, the role of necroptosis in the progression of cancer has received less attention[10,11]. Necroptosis, which is a type of regulated cell death, is triggered by diverse stimuli, including pathogen recognition receptors, cell death receptor ligands, viral RNA sensors, DNA damage, and hypoxia sensors. The culmination of these stimuli results in the formation of a supramolecular complex known as the necrosome[12]. In response to the formation of the necrosome, RIPK1 and its substrate RIPK3, along with the downstream necroptosis executor MLKL, are activated[13]. Recent evidence has shown that chemotherapy drugs and natural products can induce necroptosis[14-17]. However, whether OSW-1 induces necroptosis in tumor cells to exert anticancer effects remains unclear.

This research aimed to investigate the effect of OSW-1 on CRC cells and elucidate the mechanisms underlying necroptosis. We also conducted a tandem mass tag (TMT) proteomic analysis to predict differentially expressed proteins and possible underlying mechanisms. Our findings demonstrated that OSW-1 can indeed induce necroptosis in CRC cells. Furthermore, necroptosis triggered by OSW-1 is closely regulated by p62/SQSTM1, which is a critical regulatory


A
OSW-1
B
HT29
HCT116
Cell viability (%)
Control
1 μM
3 μM
6 μM
12 μM
24 μM
OSW-1 (1 μM)
C
HT29
HCT116
Relative LDH release
Control
OSW-1 (1 μM)
OSW-1 (2 μM)
OSW-1 (5 μM)
D1
HT29
HCT116
Colony formation (%)
Control
OSW-1 (1 μM)
D2
Control
OSW-1 (1 μM)
HT29
HCT116
E1
HCT116
HT29
Control
OSW-1 (1 μM)
PI-A
FITC-A
E2
HT29
HCT116
PI-positive cells (%)
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Mean fluorescence intensity (JC-1 red/ JC-1 green)
Control
OSW-1 (1 μM)

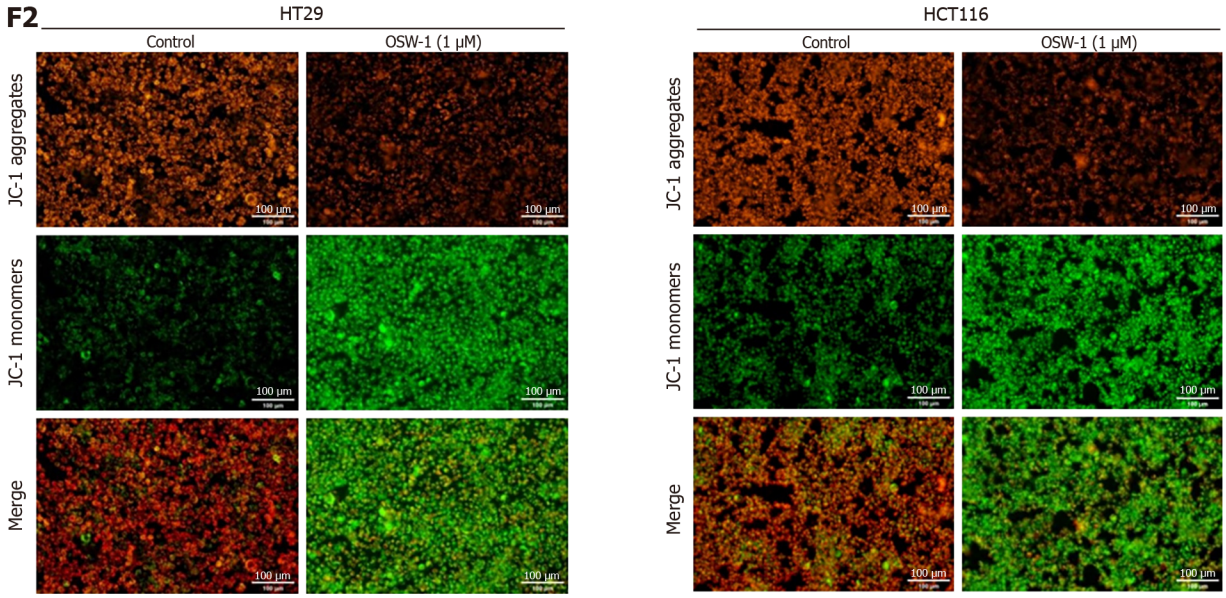


Figure 1 OSW-1 suppressed HT29 and HCT116 cell survival. A: Chemical structure of OSW-1; B: Cell counting kit-8 assay was used to access the viability of HT29 and HCT116 cells treated with different concentrations of OSW-1 for 24 h; C: Lactate dehydrogenase release assays were used to assess the cell death rate of HT29 and HCT116 cells treated with different concentrations of OSW-1 for 24 h; D: A colony formation assay was used to evaluate the clonogenic survival of HT29 and HCT116 cells following treatment with OSW-1; E: Flow cytometry with Annexin V-FITC/propidium iodide double staining was used to assess the percentage of apoptotic and necrotic HT29 and HCT116 cells after OSW-1 treatment; F: JC-1 staining was used to assess mitochondrial function in HT29 and HCT116 cells after OSW-1 treatment. *bP* < 0.01 *vs* Control, *cP* < 0.05 *vs* OSW-1 (1 μM). Each data point represents the mean ± SE. LDH: Lactate dehydrogenase; PI: Propidium iodide.

molecule in autophagy. These findings provide novel insights into the molecular mechanisms underlying necroptosis, identifying OSW-1 as a potential therapeutic drug that should be further explored.

MATERIALS AND METHODS

Cell lines and cell culture

The human HT29 (iCell-h078) and HCT116 (Procell CL-0096) CRC cell lines were obtained from iCell Bioscience (Shanghai, China) and Procell Life Science & Technology Corporation (Wuhan, China). The cells were cultured in DMEM (Gibco BRL, Invitrogen, Carlsbad, CA, United States) and McCoy's 5A medium (Gibco BRL, Invitrogen, Carlsbad, CA, United States), both supplemented with 10% fetal bovine serum (Newzerum, Christchurch, New Zealand) and 1% streptomycin and penicillin (Sigma, United States).

OSW-1 and reagents

OSW-1 (C47H68O15, purity ≥ 98%) was obtained from GlpBio Technology Company (Montclair, CA, United States). OSW-1 was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, United States) to generate a stock solution and stored at -20 °C. To achieve the desired concentrations, OSW-1 was diluted in cell culture medium, ensuring that the DMSO concentration remained below 0.1% to minimize potential adverse effects.

Cell proliferation assay

Cells (8.0×10^3/well) were seeded in 96-well plates and treated with various concentrations of OSW-1 for 24 h. A Cell Counting Kit-8 (CCK-8) assay was used to assess cell proliferation. The absorbance was measured at 450 nm with a microplate reader (Thermo Fisher Scientific, MA, United States). Data analysis was conducted utilizing GraphPad Prism 9.0 software. (GraphPad Software, La Jolla, CA, United States). Each sample was analyzed in triplicate.

Flow cytometry assay

An Annexin V-FITC/Propidium iodide (PI) apoptosis detection kit (Elabscience Biotechnology, Wuhan, China) was used to detect cell apoptosis. After treating cells with OSW-1 for 24 h, the cells were digested to generate single-cell suspensions, which were stained according to the kit instructions. The experiment was replicated three times for each measurement.

Mitochondrial membrane potential assay

Cells were seeded in a six-well plate and washed with PBS (Procell, Wuhan, China) after OSW-1 treatment for 24 h. JC-1 working solution (Elabscience Biotechnology, Wuhan, China) was added to the cells, which were then incubated at 37 °C for 30 minutes and washed with PBS. Each sample was analyzed in triplicate.

Table 1 Quantitative real-time-PCR primer sequences

Primer	Sequences (5'-3')
p62-F	5'-CTGGGACTGAGAAGGCTCAC-3'
p62-R	5'-GCAGCTGATGGTTTGGAAAT-3'
LC3-F	5'-AGCAGCATCCAACCAAAATC -3'
LC3-R	5'-CTGTGTCCGTTCACCAACAG -3'
RIPK1-F	5'-GGGAAGGTGTCTCTGTGTTTC-3'
RIPK1-R	5'-CCTCGTTGTGCTCAATGCAG-3'
β-actin-F	5'-AGTTGCGTTACACCCTTTCTTG-3'
β-actin-R	5'-GCTGTCACCTTCACCGTTCC-3'

Hoechst 33342/PI staining assay

Cells were seeded in a six-well plate and washed with PBS after OSW-1 treatment for 24 h. Hoechst 33342/PI working solution was added, and the samples were incubated for 20 min in the dark. Each sample was analyzed in triplicate.

Lactate dehydrogenase release assay

Lactate dehydrogenase (LDH) assay kit was used to measure LDH release and determine LDH activity (Beyotime Biotechnology, Shanghai, China). After treatment with OSW-1 for 24 h, 200 μL of LDH working solution was added to the cells and incubated for 30 min at room temperature according to the manufacturer's instructions. The absorbance at was measured 450 nm with a microplate reader. Each sample was analyzed in triplicate.

Proteomic analysis

To assess changes in protein levels, a 6-plex TMT proteomic assay was employed. The proteomic experiments included two groups: The control group (treated with DMSO) and the OSW-1 treatment group. CRC cells were exposed to 1 μM OSW-1 for 24 h, and each group included three independent samples. Sangon Biotech (Shanghai, China) conducted the proteomic profiling, including enzymatic hydrolysis, labeling, mass spectrometry, and bioinformatics analysis, which included Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses.

Cell morphology observation

Cells (5.0×10^5/well) were seeded in a six-well plate. The cells were exposed to OSW-1 for 24 h and then observed and photographed by optical microscopy (Nikon, Tokyo, Japan) to determine cell morphology.

Transmission electron microscopy images

CRC cells were plated on a sterile cover glass within a Petri dish. After treatment with OSW-1 for 24 h, the slides were gently washed with PBS, followed by incubation with an electron microscopy fixative (Servicebio, Wuhan, China) in the Petri dish. After the samples were fixed for 2 h at room temperature, the Petri dishes were transferred to 4 °C for storage. Specimens were affixed to cuprum grids, dehydrated with ethanol, and incubated with 2.6% lead citrate, after which exposure to CO_2 was avoided for 8 min. Following desiccation with filter paper, the copper grids were placed on the grid board and allowed to air-dry overnight. Subsequently, the cell morphology of the samples was examined using transmission electron microscopy (TEM; HITACHI, Tokyo, Japan).

Quantitative real-time PCR and RNA interference

CRC cells were exposed to OSW-1 for 24 h, and PCR was carried out using an ABI 7500 PCR instrument and the SYBR Green Premix Pro Taq HS quantitative real-time PCR Kit (TaKaRa Biotechnology, Beijing, China). Relative mRNA expression levels were determined by normalization to the mRNA expression level of β-actin using the $2^{-\Delta\Delta Ct}$ method (Table 1). The experiments were performed in triplicate. Downregulation of p62/SQSTM1 in CRC cells was achieved using the following siRNA duplex from GenePharma: CAUCCAGUAUUCAAAGCAUTT and AUGCUUUGAAUACUG-GAUGTT. Cells were transfected using the GP-transfect-Mate Kit (Genepharma Company, Suzhou, China).

Western blotting

After being treated with OSW-1 for 24 h, proteins were extracted from total cellular lysates using RIPA buffer (Proteintech, Wuhan, China), and the protein contents were quantified with a BCA protein assay kit (Elabscience, Wuhan, China). The protein samples were electrophoresed on a 10% SDS-PAGE gel (Severbio, Beijing, China) and electrotransferred to PVDF membranes (Millipore, MA, United States). The membranes were blocked at room temperature for 30 min and subsequently incubated with primary antibodies at 4 °C overnight. Then, secondary antibodies were added, and the immunoreactive bands were observed using enhanced chemiluminescence. The western blot bands were evaluated using ImageJ software.

Coimmunoprecipitation

According to the manufacturer's instructions, IP was performed with protein G magnetic beads after cells had been treated with OSW-1 for 24 h. After cells were lysed in RIPA buffer, the proteins were collected from the supernatants and incubated at 4 °C overnight with anti-RIPK1 (5 mg). To prepare a complex between the antibody and antigen, a mixture of protein G magnetic beads (60 μL) was added, and the mixture was incubated. The immunomagnetic bead-antibody-antigen complex was separated using a magnetic separator. After three washes of the beads with PBS, Western blotting was used to detect endogenous interactions between the specific antibody and other proteins.

Immunofluorescence assay

Cells were cultured in confocal dishes (BIOFIL, Guangzhou, China) and stimulated with OSW-1 for 24 h. Subsequently, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature. A permeabilization buffer of 0.5% Triton-X 100 was used, followed by blocking with a solution containing PBS, 5% goat serum, and 0.5% Triton-X 100 at room temperature for 30 min. Primary antibodies, including anti-RIPK1 and anti-p62 antibodies, were diluted in a primary antibody diluent (5% goat serum), added and incubated overnight at 4 °C. After washing with PBS, secondary antibodies (goat anti-rabbit Cy3 and goat anti-mouse FITC) were added, and the samples were incubated for 1 h at room temperature. Then, the samples were subjected to three consecutive washes with PBS. Finally, the nuclei were stained with DAPI. Observations were conducted at 40 × magnification with a 0.75 × zoom lens (Leica, Germany). Spectral Borealis lasers (green, 488 nm; red, 561 nm; blue, 405 nm) were used for excitation. A series of images were acquired with Leica SP8 software.

Autophagic flux analysis

Images were captured to analyze the cellular autophagic flux using a confocal laser scanning microscope (Leica, Germany). For quantitative assessment of fluorescence intensity, ImageJ software was used to measure the integral optical density.

Xenograft tumor animal models

Four-week-old BALB/c-nude male mice were obtained from the Model Animal Research Institute of Dalian Medical University. A subcutaneous tumor model was established by injecting HT29 cells under the skin of each nude mouse. When the tumor volume reached 50 mm^3, the nude mice were randomly divided into three groups: (1) The control group (treated with DMSO), (2) the low-dose OSW-1 group (0.01 μg/kg daily), and (3) the high-dose OSW-1 group (1 μg/kg daily). Tumor growth was monitored twice a week by measurements taken with a digital caliper. For further analysis, the mice were sacrificed humanely at 14 d, and the tumors were isolated. Tumor volumes were calculated with the following formula: V (mm^3) = L (mm) × W^2 (mm^2) × 0.5. All the animal procedures and protocols were approved by the Committee on the Ethics of Animal Experiments of Dalian Medical University, No. AEE22108.

Immunohistochemistry

The paraffin sections were dewaxed using prewarmed xylene and dehydrated with an alcohol gradient. An appropriate amount of peroxidase blocking agent (Zsbio, Beijing, China) was added, followed by incubation at room temperature for 10 min. Subsequently, primary antibodies (Proteintech, Wuhan, China) were applied, and the samples were incubated overnight at 4 °C in a refrigerator. After overnight storage, a secondary antibody was added. The sections were then subjected to DAB staining and restained with hematoxylin. Finally, the slides were examined under a microscope.

Statistical analysis

All the graphs were generated using GraphPad Prism v.9 software. The data are presented as the mean ± SD and were analyzed using SPSS 23.0 software. One-way analysis of variance was used for data analysis, with statistical significance set at $P < 0.05$.

RESULTS

OSW-1 inhibited the proliferation of CRC cells

To investigate the ability of OSW-1 to suppress cell survival, we conducted a CCK-8 assay to assess the viability of CRC cells treated with different concentrations of OSW-1 for 24 h. The cytotoxicity results from the CCK-8 assay revealed a dose-dependent effect of OSW-1 on the viability of CRC cells, as shown in Figure 1B. Notably, there was a significant decrease in cell viability in the treated group compared to the control group. Furthermore, to determine the cell death rates, LDH release assays were conducted (Figure 1C). The results indicated a noticeable increase in LDH release with increasing OSW-1 concentration. OSW-1 (1 μM) significantly inhibited CRC cell survival, which was consistent with the CCK-8 results. Therefore, 1 μM OSW-1 was used as the concentration for the subsequent studies.

Moreover, a colony formation assay was performed to assess the effect of OSW-1 on colony formation ability, and the results revealed the significant inhibition of CRC cell colony formation (Figure 1D). As shown in Figure 1E, dual-fluorescence staining with Annexin V-FITC/PI demonstrated that CRC cells treated with OSW-1 underwent necrosis, as indicated by a notable increase in the percentage of necrotic and late apoptotic cells. To further investigate the potential mechanism underlying necrosis, the membrane-permeable fluorescent probe JC-1 was utilized. Under normal conditions, JC-1 aggregates in healthy cells. However, necroptosis can disrupt the mitochondrial membrane potential (Δψm), leading

to the conversion of JC-1 aggregates (red) into monomers (green). We found that OSW-1 is a potent inducer of necroptosis that can cause JC-1 fluorescence to shift from red to green in CRC cells, which is indicative of a decrease in the Δψm (Figure 1F).

Proteomic analysis of the mechanism underlying the antitumor effects of OSW-1

Our proteomic analysis revealed distinct patterns of protein expression between the OSW-1 treatment group and the control group (Figure 2A). After applying the criteria of a fold change > 1.2 and a significance level of $P < 0.05$ for filtering, our analysis identified 312 differentially expressed proteins in the OSW-1 treatment group. Among these, 143 were associated with the cell membrane, 114 with the endomembrane system, 83 with the cell periphery, 63 with the extracellular region, and 55 with the intrinsic component of the membrane (Figure 2B). A volcano plot was constructed, which shows 186 proteins with decreased expression and 126 proteins with increased expression in the OSW-1 treatment group (Figure 2C). To comprehensively understand the functional roles of proteins that were affected by OSW-1, we conducted enrichment analyses of GO terms, KEGG pathways, and protein domains. Our GO enrichment analysis (Figure 2D) revealed that the proteins that were affected by OSW-1 were predominantly associated with biological processes related to cell communication. Additionally, regarding cellular components, these proteins were enriched mainly in the cell membrane. Additionally, our analyses of KEGG pathway enrichment revealed that OSW-1 induced significant changes in the expression of proteins related to the necroptosis pathway (Figure 2E). Collectively, these findings suggest that OSW-1 may attenuate CRC development by modulating signaling pathways related to necroptosis.

OSW-1 triggers necroptosis in cultured CRC cells

Subsequently, we observed morphological changes in CRC cells following exposure to OSW-1 under an optical microscope (Figure 3A). After OSW-1 exposure, CRC cells exhibited a discernible change in their original morphology, displaying signs of swelling and disruption, which were indicative of classic necrosis. As shown in Figure 3B, TEM images revealed typical necroptotic morphological changes in CRC cells after treatment with OSW-1. These changes included the appearance of swollen cells, irregular nuclear chromatin, membrane rupture, and cytoplasmic extravasation.

Since RIPK1, RIPK3, and MLKL are important markers of necroptosis, our investigation examined the protein levels of these three necroptosis-associated molecules in OSW-1-treated CRC cells by western blotting analysis. As shown in Figure 3C, phosphorylated RIPK1, which serves as a marker of RIPK1 activation, was clearly increased in CRC cells following OSW-1 treatment. Additionally, in addition to phosphorylating RIPK3, which is the binding partner of RIPK1, OSW-1 also increased the phosphorylation of RIPK3 in CRC cells. Furthermore, OSW-1 equally enhanced the phosphorylation of MLKL, which is the downstream necroptosis executor in CRC cells. To further characterize necroptosis, we performed a Hoechst 33342/PI double staining assay. The results (Figure 3D) demonstrated a noticeable increase in the proportion of dead cells after OSW-1 treatment. These data strongly suggested that necroptosis is triggered by OSW-1 in CRC cells.

OSW-1 induced necroptosis in CRC cells through the RIPK1/RIPK3/MLKL pathway

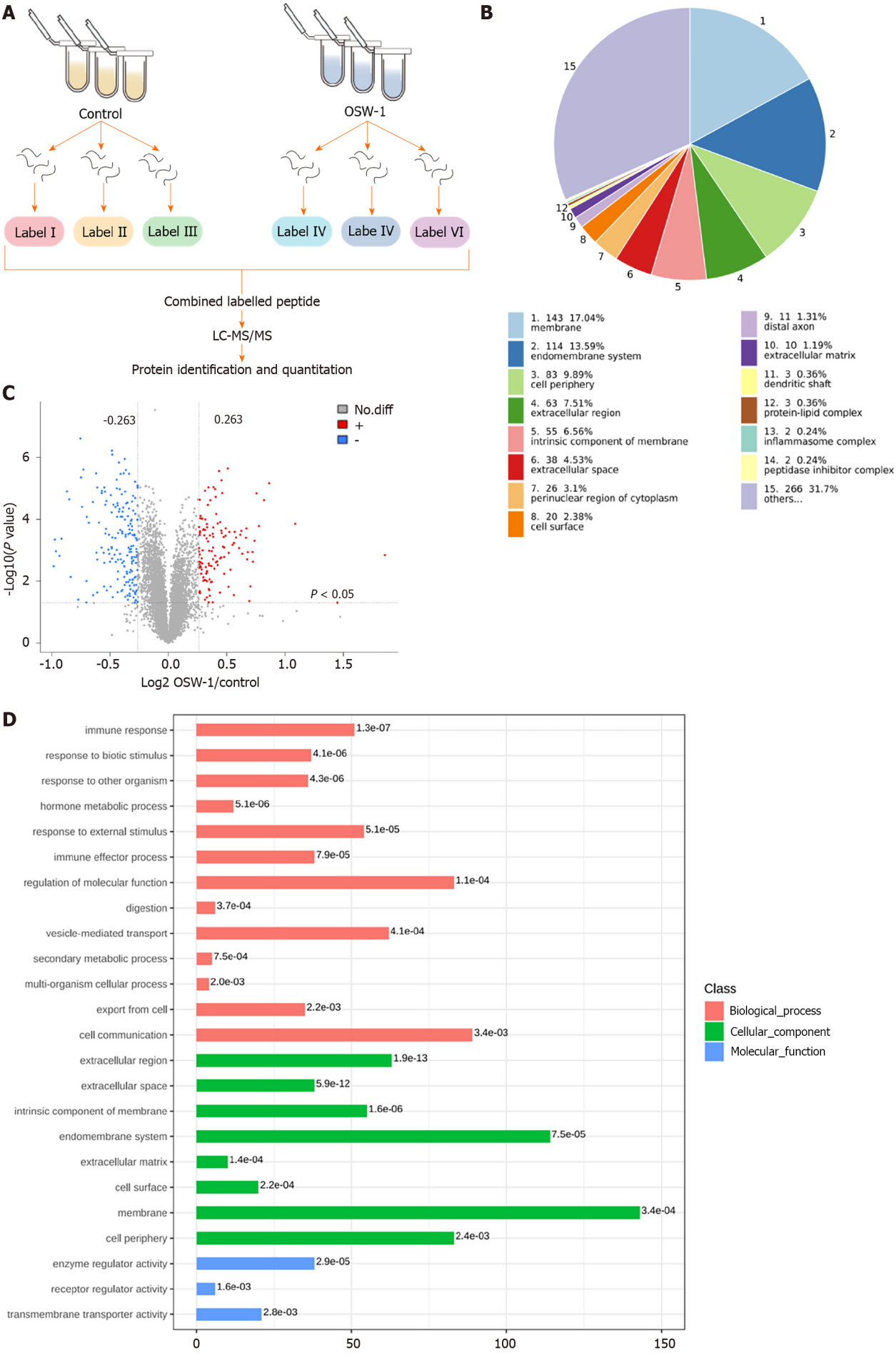
In this study, we focused on determining whether OSW-1-induced necroptosis requires the RIPK1/RIPK3/MLKL pathway, which is to be a critical pathway in necroptosis. The initial small-molecule inhibitor of RIPK1, necrostatin-1 (Nec-1), was shown to trigger necroptosis in cells. Since RIPK1 serves as a critical mediator of necroptosis, Nec-1 has been widely used to analyze this cellular process. To investigate the potential molecular mechanism underlying OSW-1-mediated necroptosis, we evaluated the impact of various Nec-1 concentrations on cell viability in cell culture experiments. The results revealed a noticeable increase in cell viability with increasing Nec-1 concentration (Figure 4A). Treatment with 10 μM Nec-1 significantly enhanced the survival of CRC cells, so this concentration was chosen for subsequent experiments.

To further explore the possible signaling pathways related to necroptosis, we investigated RIPK1, RIPK3, and MLKL in CRC cells treated with OSW-1 and Nec-1 *via* western blotting analysis. In combination with OSW-1, Nec-1 significantly inhibited both RIPK3 activation and MLKL activation as well as the subsequent necroptosis induced by OSW-1 (Figure 4B). Moreover, there was a distinct shift from JC-1 fluorescence from red to green when the cells were exposed to OSW-1. However, in combination with Nec-1, the intensity of green fluorescence significantly decreased (Figure 4C). In addition, Hoechst 33342/PI staining demonstrated a notable increase in the proportion of live cells after treatment with both OSW-1 and Nec-1 (Figure 4D). Moreover, the LDH release assay revealed a significant decrease in LDH release after treatment with both OSW-1 and Nec-1 (Figure 4E).

Based on our data, OSW-1 induces necroptosis in a manner that requires the activation of the RIPK1/RIPK3/MLKL pathway.

Impairment of the autophagic flux results in the accumulation of p62/SQSTM1, which facilitates OSW-1-induced necroptosis through its interaction with RIPK1

Based on the findings from the proteomic analysis, we focused on a notable protein, p62/SQSTM1, which is to be involved in the autophagic flux. According to previous studies, p62/SQSTM1 directly interacts with RIPK1. The region that is responsible for the binding of p62/SQSTM1 to RIPK1 is the ZZ structural domain (amino acids 122-167). We hypothesized that p62/SQSTM1 might function as a signaling platform and be involved in OSW-1-induced RIPK1-dependent necroptosis in CRC cells. Our primary objective was to test this hypothesis by assessing the protein expression of p62/SQSTM1 in CRC cells. Strikingly, the results showed significant upregulation of p62/SQSTM1 expression in the OSW-1 treatment group compared to the control group (Figure 5A). Our findings indicate that exposure of CRC cells to OSW-1 Leads to the accumulation of the p62/SQSTM1 proteins, which is generally associated with impaired autophagic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Control
OSW-1
Label I
Label II
Label III
Label IV
Labe IV
Label VI
Combined labelled peptide
LC-MS/MS
Protein identification and quantitation
B
1. 143 17.04% membrane
2. 114 13.59% endomembrane system
3. 83 9.89% cell periphery
4. 63 7.51% extracellular region
5. 55 6.56% intrinsic component of membrane
6. 38 4.53% extracellular space
7. 26 3.1% perinuclear region of cytoplasm
8. 20 2.38% cell surface
9. 11 1.31% distal axon
10. 10 1.19% extracellular matrix
11. 3 0.36% dendritic shaft
12. 3 0.36% protein-lipid complex
13. 2 0.24% inflammasome complex
14. 2 0.24% peptidase inhibitor complex
15. 266 31.7% others...
C
No.diff
+
-
-0.263
0.263
P < 0.05
-Log10(P value)
Log2 OSW-1/control
D
immune response 1.3e-07
response to biotic stimulus 4.1e-06
response to other organism 4.3e-06
hormone metabolic process 5.1e-06
response to external stimulus 5.1e-05
immune effector process 7.9e-05
regulation of molecular function 1.1e-04
digestion 3.7e-04
vesicle-mediated transport 4.1e-04
secondary metabolic process 7.5e-04
multi-organism cellular process 2.0e-03
export from cell 2.2e-03
cell communication 3.4e-03
extracellular region 1.9e-13
extracellular space 5.9e-12
intrinsic component of membrane 1.6e-06
endomembrane system 7.5e-05
extracellular matrix 1.4e-04
cell surface 2.2e-04
membrane 3.4e-04
cell periphery 2.4e-03
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receptor regulator activity 1.6e-03
transmembrane transporter activity 2.8e-03
Class
Biological_process
Cellular_component
Molecular_function

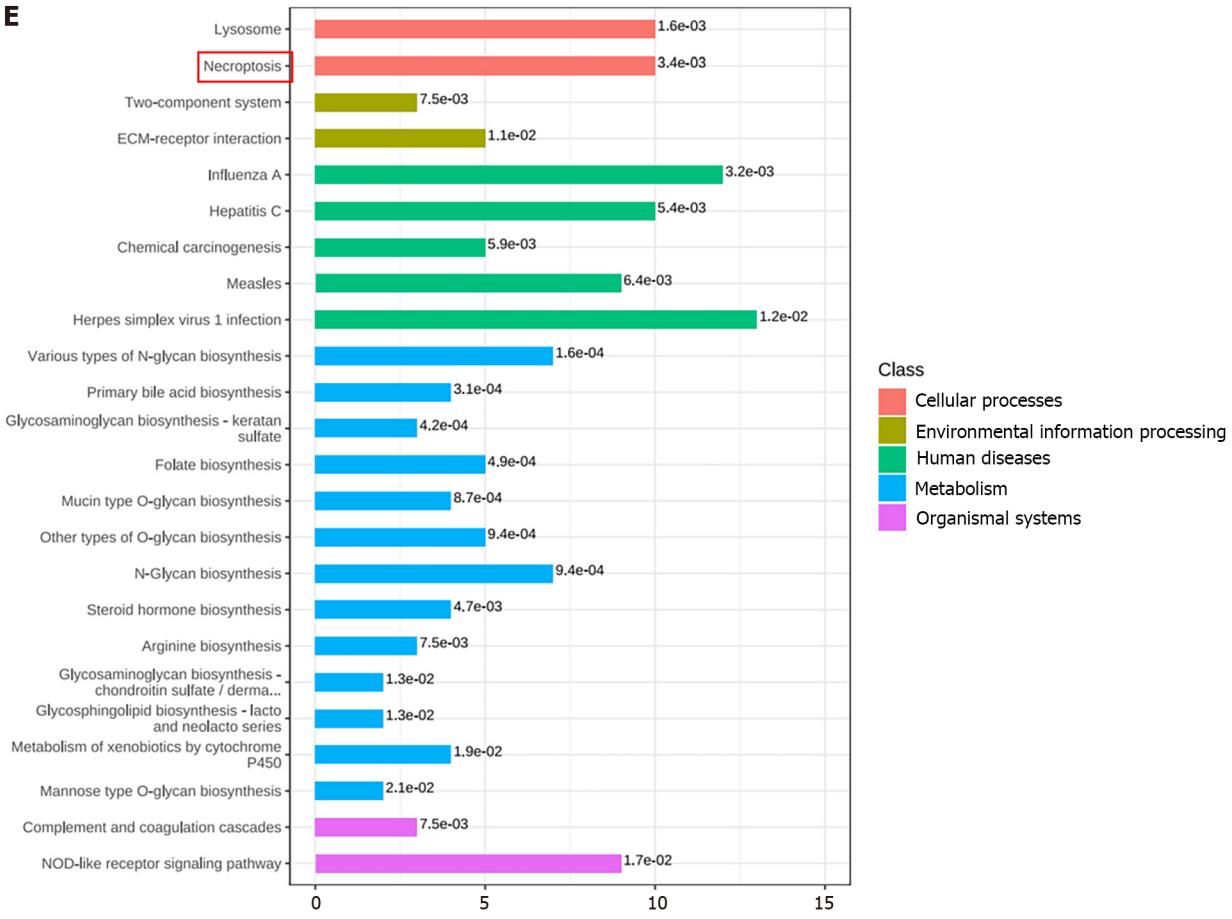


Figure 2 Quantitative proteomic analysis was also conducted on control and OSW-1-treated colorectal cancer cells, and bioinformatic analyses were subsequently performed on the differentially expressed proteins. A: Schematic diagram outlining the process of proteomic analysis in this study; B: Subcellular localization of proteins that were altered by OSW-1; C: Volcano plot showing OSW-1-induced changes in proteins; red dots indicate increased proteins, and blue dots indicate decreased proteins; D: The top 20 enriched gene ontology (GO) terms were identified using Fisher's exact test for the biological process, molecular function, and cellular component categories. The vertical axis shows the GO terms in each category, while the horizontal axis shows the protein number for each item. The numbers beside the bars indicate enrichment factors, indicating the significance and reliability of the proteins enriched in each item; E: Enriched Kyoto Encyclopedia of Genes and Genomes pathways associated with the differentially expressed proteins, with the numbers beside the bars representing the *P* value calculated using Fisher's exact test.

degradation of p62/SQSTM1-bound substrates. We suspected that the upregulation of p62/SQSTM1 during OSW-1-induced necroptosis might be a result of impairment of the autophagic flux. Therefore, we assessed the protein levels of LC3-II, a crucial indicator of autophagy. These findings suggested that the level of LC3-II was increased after OSW-1 treatment in comparison to that in the control group (Figure 5A).

Additionally, the mRNA levels of p62/SQSTM1 and LC3 were elevated in CRC cells treated with OSW-1 (Figure 5B). These results suggested a connection between the aggregation of p62/SQSTM1 and the inhibition of autophagy-driven protein degradation. To verify this concept, we employed recombinant adenoviral vectors encoding GFP and mCherry-tagged LC3 (Ad-GFP & mCherry-LC3) to assess the autophagic flux. As shown in Figure 5C, OSW-1 progressively decreased the number of red dots (mCherry puncta lacking GFP fluorescence, indicative of autolysosomes) while increasing the number of yellow dots (overlapping mCherry with GFP fluorescence puncta, indicative of autophagosomes). These findings suggest that OSW-1 can impair the autophagic flux, contributing to the accumulation of p62/SQSTM1.

To explore the regulatory effects of p62/SQSTM1 on the RIPK1-related pathway, we conducted coimmunoprecipitation and immunofluorescence colocalization experiments. As shown in Figure 5D, the coimmunoprecipitation experiment further confirmed that OSW-1 promotes the interaction between RIPK1 and p62/SQSTM1, demonstrating the formation of a complex between p62/SQSTM1 and RIPK1. Moreover, immunocytochemical staining for p62/SQSTM1 (green) and RIPK1 (red) and analysis by confocal fluorescence microscopy revealed notable colocalization (yellow) of p62/SQSTM1 and RIPK1 compared to that in the control group. Following Nec-1 treatment, we observed a significant reduction in the colocalization of p62/SQSTM1 and RIPK1 compared to that in the OSW-1 group (Figure 5E).

To assess the role of p62/SQSTM1 in necroptosis, p62/SQSTM1 siRNA was transduced into CRC cells to reverse the effect of p62/SQSTM1 overexpression on necroptosis. When CRC cells that were transiently transfected with the control construct were exposed to OSW-1, they exhibited a reduction in p62/SQSTM1 expression (Figure 6A). Additionally, silencing p62/SQSTM1 Led to decreased levels of RIPK1 in CRC cells treated with OSW-1 (Figure 6A). Notably, RIPK1 expression was significantly decreased after specific siRNA-mediated knockdown of p62/SQSTM1 in CRC cells,

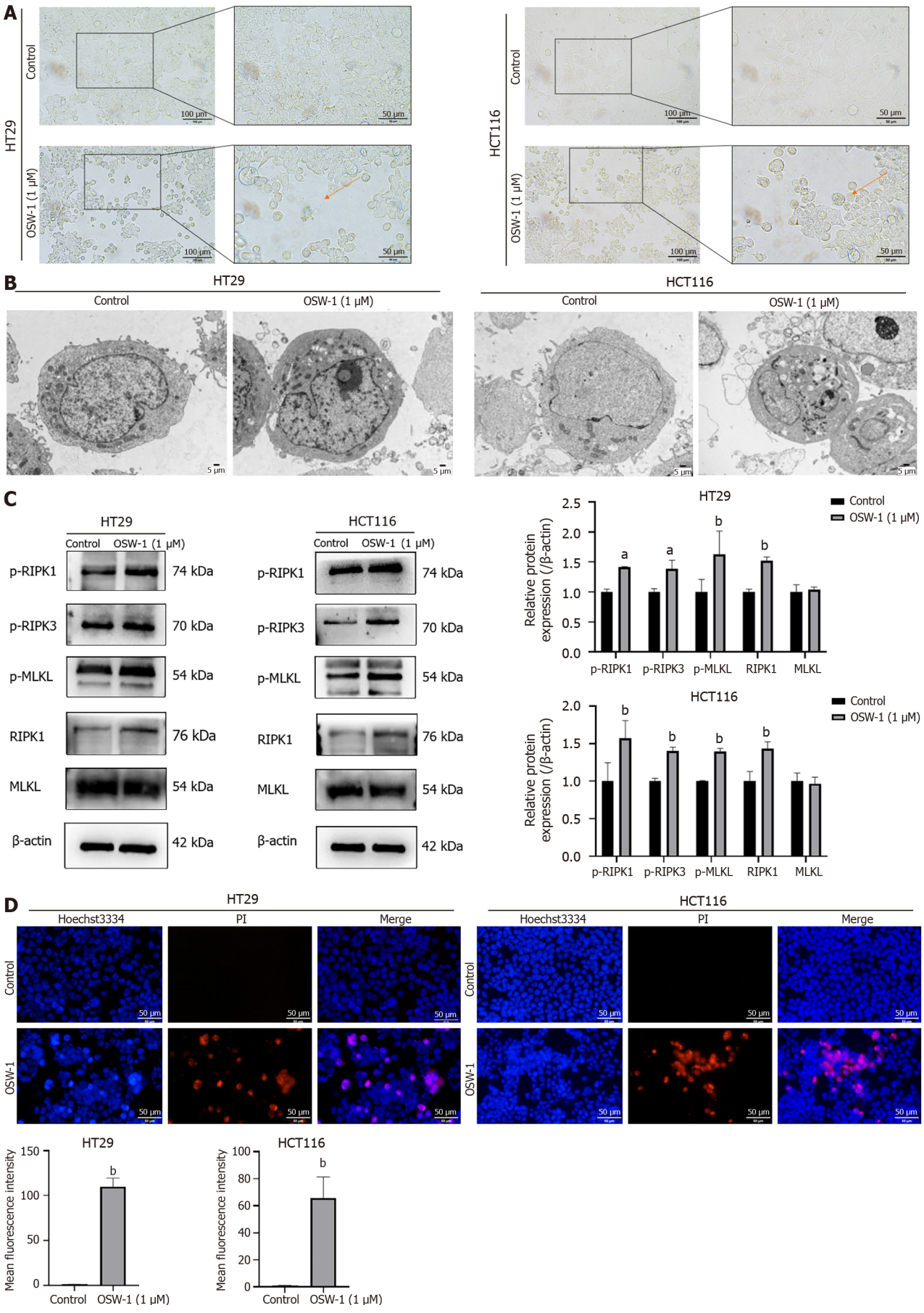


Figure 3 OSW-1 triggers necroptosis in colorectal cancer cell culture. A: Necrotic morphological changes were observed in HT29 and HCT116 cells treated with OSW-1 using optical microscopy (400 × magnification). Necrotic cells are indicated by red arrows; B: Typical morphological changes associated with

necroptosis were identified in HT29 and HCT116 cells treated with OSW-1 through TEM (3000 × magnification); C: The expression levels of proteins associated with necroptosis in HT29 and HCT116 cells following 24 h of exposure to OSW-1; D: A Hoechst 33342/propidium iodide dual staining assay was used to assess the rate of necroptosis in HT29 and HCT116 cells following treatment with OSW-1. Scale bar = 50 μm. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* Control. Each data point represents the mean ± SE. PI: Propidium iodide.

suggesting that p62/SQSTM1 protects RIPK1 from degradation. To further elucidate the role of p62/SQSTM1 in OSW-1-induced necroptosis, western blotting experiments were conducted on CRC cells. The results indicated that the expression of phosphorylated RIPK1, phosphorylated RIPK3, and MLKL was decreased upon the silencing of p62/SQSTM1 (Figure 6B). Furthermore, Hoechst 33342/PI staining showed a notable increase in the proportion of live cells, indicating the restoration of necroptosis in p62/SQSTM1 siRNA-transfected cells (Figure 6C). These findings highlight the crucial role of p62/SQSTM1 in OSW-1-induced necroptosis in CRC cells.

In summary, our results demonstrated that impairment of the autophagic flux leads to the accumulation of p62/SQSTM1, which increases necroptosis in OSW-1-treated CRC cells *via* interaction with RIPK1 and phosphorylation of RIPK1/RIPK3/MLKL pathway-related proteins.

Inhibition of CRC cell proliferation is associated with OSW-1-induced necroptosis in vivo

To assess the antitumor effects of OSW-1 on CRC *in vivo*, we established a mouse xenograft model. Following the development of palpable tumors, the mice were administered OSW-1 at doses of 0.01 μg/kg and 1 μg/kg for 14 d (Figure 7A). As expected, OSW-1 significantly suppressed the growth of tumors (Figure 7B). The tumor volumes in mice treated with OSW-1 were consistently smaller than those in control mice, with the high-dose group (1 μg/kg) displaying notably reduced tumor sizes compared to those in the low-dose group (0.01 μg/kg; Figure 7C). Moreover, the tumor weight exhibited a similar trend (Figure 7C). Furthermore, the influence of OSW-1 on CRC was assessed through immunohistochemistry (IHC). Compared with those in the control group, the expression levels of the necroptosis-related proteins p-RIPK1 and p-MLKL increased with increasing OSW-1 concentration (Figure 7D). These findings were consistent with the results of the *in vitro* experiments. Furthermore, the qRT-PCR results revealed a concentration-dependent increase in the expression of RIPK1 and MLKL in response to OSW-1 treatment (Figure 7E), which was consistent with the IHC results. In summary, our results suggest that OSW-1 suppresses tumor growth in a mouse xenograft model by activating the necroptosis pathway.

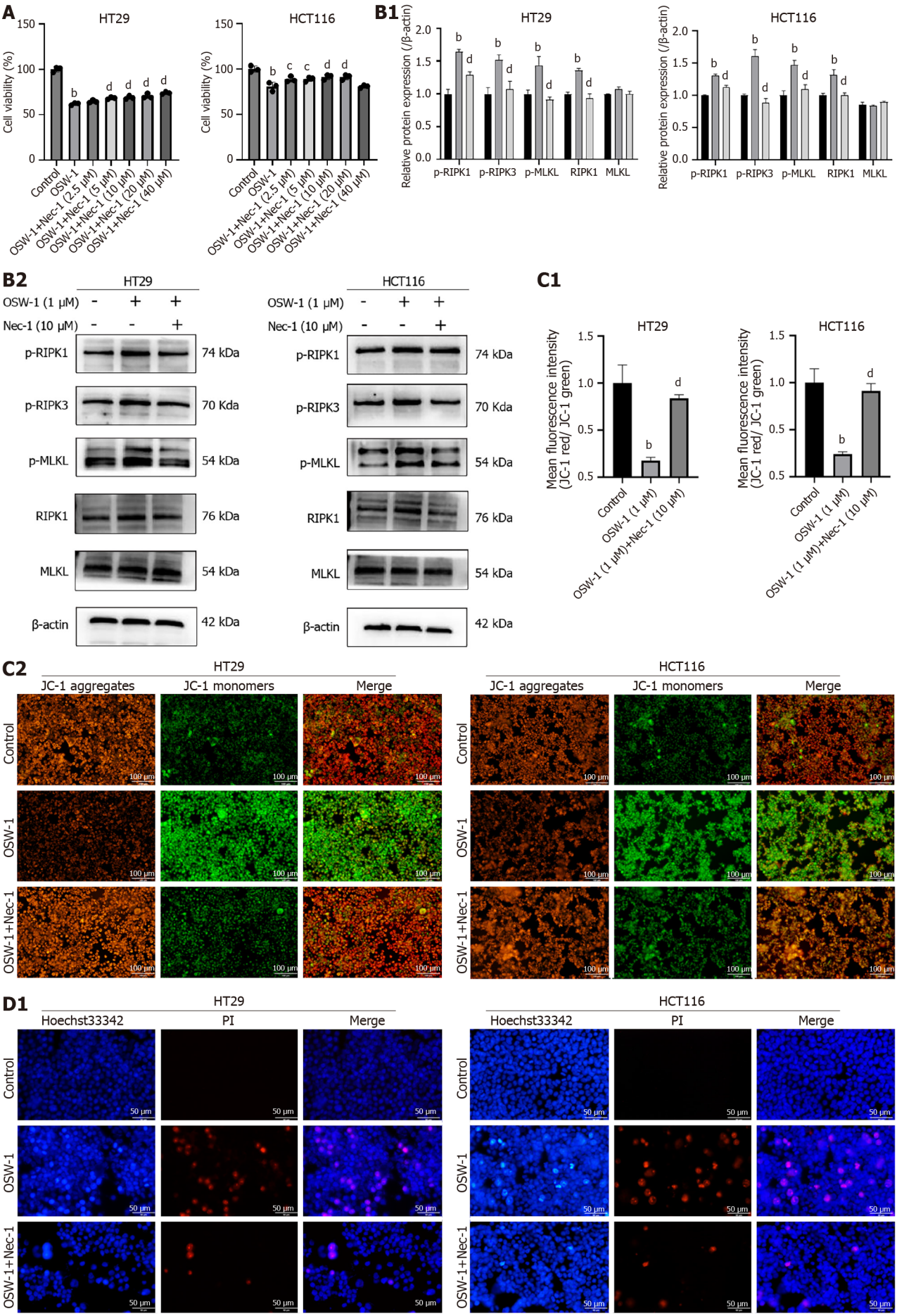
DISCUSSION

CRC is a heterogeneous malignant tumor that poses a significant threat to human survival[18-20]. Its pathogenic factors are complex and include gene mutation, age, family history, obesity, and physical inactivity[21,22]. With better understanding of CRC, the spectrum of treatment options has expanded, including endoscopic and surgical excision, radiotherapy, immunotherapy, and targeted therapy[23-25]. These treatments have led to a substantial reduction in cancer progression and an increased survival rate. However, despite these advancements, CRC remains the second most common cause of cancer-related death worldwide, primarily due to challenges encountered in clinical trials, such as resistance to radiotherapy and chemotherapy that aim to induce apoptosis[26,27]. To develop a novel strategy for preventing CRC progression, exploring alternative mechanisms of cell death in CRC by integrating high-throughput proteomics with conventional molecular techniques is imperative.

The natural product OSW-1, which is derived from plants, can selectively kill cancer cells[28]. In recent years, OSW-1 has attracted considerable attention due to its potent and selective cytotoxic effects against various types of cancer cell lines, suggesting a potentially novel mechanism of action. In the early stages of research, ovarian granulosa cell proliferation and the expression of the steroidal enzyme were thought to be inhibited by OSW-1[29]. In a previous study, OSW-1 was shown to exert cytotoxic effects against diverse cancer cells, including leukemia cells, and higher toxicity was observed against malignant cells than against normal cells[30]. However, the specific mechanism underlying the action of OSW-1 has not been elucidated. Our study revealed that OSW-1 effectively inhibited proliferation and suppressed survival in CRC cells.

Quantitative proteomic methods are widely utilized due to their ability to reveal the dynamics of protein expression and interactions on a global scale. This approach significantly contributes to comprehending gene functions and cellular processes. In this study, we employed a proteomic approach to identify proteins whose expression was altered in response to OSW-1 treatment. A total of 312 proteins exhibited differential expression. Considering the substantial number of differentially expressed proteins and the high enrichment factor, we focused our attention on the necroptosis pathway for further mechanistic exploration.

In recent years, necroptosis, which is a novel form of PCD, has emerged as an essential form of cell death that contributes to various diseases, and its role in tumors has received particular attention. Moreover, a large amount of evidence has shown that necroptosis can impact tumor development, rendering necroptosis as an important area of interest in cancer treatment[31-34]. Notably, the relationship between necroptosis and gastric cancer has been established [35]. Moreover, RIPK1, which is a pivotal signaling node in necroptosis, has been shown to cooperate with TRAF2 to suppress murine and human hepatocarcinogenesis[36]. Recent research has highlighted the ability of several chemotherapeutic drugs and natural products to induce necroptosis and inhibit tumor growth. For instance, jaceosidin has been identified as an inducer of necroptosis in human glioblastoma multiforme, suggesting its potential as a therapeutic agent


A
HT29
HCT116
Cell viability (%)
Control
OSW-1
OSW-1+Nec-1 (2.5 μM)
OSW-1+Nec-1 (5 μM)
OSW-1+Nec-1 (10 μM)
OSW-1+Nec-1 (20 μM)
OSW-1+Nec-1 (40 μM)
B1
Relative protein expression (/β-actin)
p-RIPK1
p-RIPK3
p-MLKL
RIPK1
MLKL
B2
OSW-1 (1 μM)
Nec-1 (10 μM)
74 kDa
70 Kda
54 kDa
76 kDa
42 kDa
β-actin
C1
Mean fluorescence intensity (JC-1 red/ JC-1 green)
OSW-1 (1 μM)
OSW-1 (1 μM)+Nec-1 (10 μM)
C2
JC-1 aggregates
JC-1 monomers
Merge
100 μm
D1
Hoechst33342
PI
50 μm

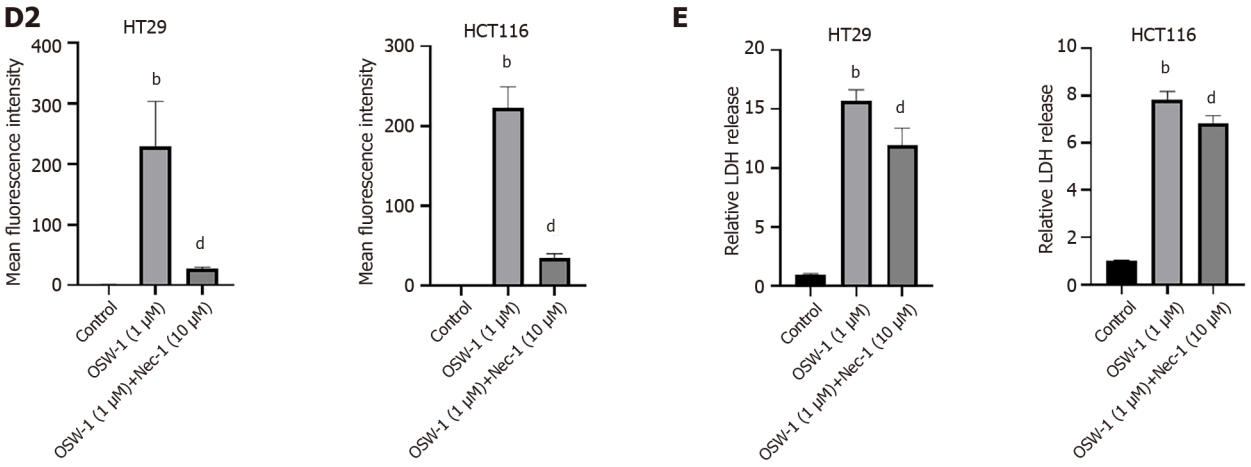


Figure 4 OSW-1 induced necroptosis in colorectal cancer cells through the RIPK1/RIPK3/MLKL pathway. A: Cell Counting Kit-8 assay was used to assess the viability of HT29 and HCT116 cells treated with OSW-1 and different concentrations of necrostatin-1 (Nec-1) for 24 h; B: The expression levels of necroptosis-related proteins in HT29 and HCT116 cells after exposure to OSW-1 for 24 h with the addition of Nec-1; C: Assessment of mitochondrial function *via* JC-1 staining of HT29 and HCT116 cells after exposure to OSW-1 for 24 h with the addition of Nec-1; D: A Hoechst 33342/PI dual staining assay was used to examine cell necroptosis after 24 h of treatment with OSW-1 and Nec-1. Scale bar = 50 μm; E: Lactate dehydrogenase release was used to assess the cell death rate of HT29 and HCT116 cells exposed to OSW-1 and Nec-1 for 24 h. $^{b}P < 0.01$ *vs* Control, $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* OSW-1. Each data point represents the mean ± SE. PI: Propidium iodide; Nec-1: necrostatin-1.

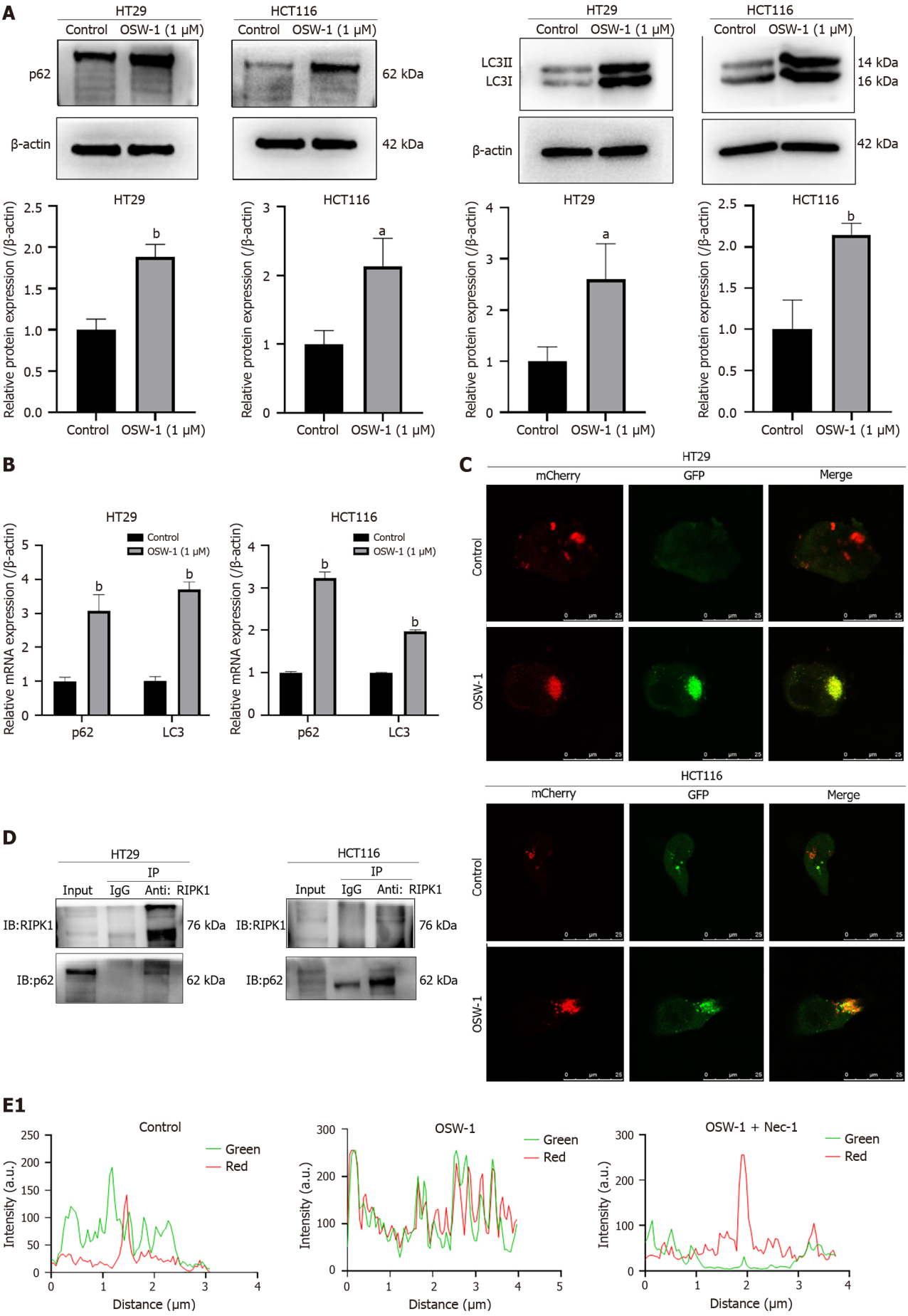
for patients[37]. Additionally, the arborinane triterpene compound 3-O-acetylrubianol C, which is isolated from the Rubia Philippines, has been found to promote tumor necrosis factor-triggered necroptotic cell death[38].

Our findings indicate that OSW-1 effectively inhibited the proliferation of CRC cells. Moreover, the percentage of necrotic cells increased, as shown by Annexin V-FITC/PI dual-fluorescence staining, indicating the occurrence of necrosis. The change in the Δψm further confirmed the role of OSW-1 as an inducer of necroptosis. Our findings indicate that OSW-1 effectively triggered necroptosis in CRC cells. More strikingly, optical microscopy and TEM revealed morphological changes that are typical of necroptosis, such as membrane rupture and cytoplasmic vesiculation, in CRC cells. Additionally, a Hoechst 33342/PI double staining assay was used to characterize necroptosis. These data strongly suggested the occurrence of necroptosis in CRC cells following OSW-1 treatment.

The RIPK1/RIPK3/MLKL pathway Is widely recognized as the classic pathway that regulates necroptosis under diverse conditions[39]. In response to stimuli such as ischemia/reperfusion, inflammation, and certain medicines, RIPK1 binds to RIPK3 to form a necrosome, which triggers MLKL activation and translocation, ultimately resulting in cell lysis. This study utilized the RIPK1-specific inhibitor Nec-1 to explore whether OSW-1 induces necroptosis in CRC cells through the classical necroptotic pathway. Nec-1 demonstrated marked proliferative effects and inhibited cell death in CRC, promoting cell survival. Western blotting analysis revealed significant upregulation of p-RIPK1, p-RIPK3, and p-MLKL expression in CRC cells treated with OSW-1. However, Nec-1 effectively attenuated the increases in these protein levels. In addition, consistent with these findings, Hoechst 33342/PI staining indicated a substantial increase in the proportion of live cells after combined treatment with OSW-1 and Nec-1 compared to treatment with OSW-1 alone. Furthermore, the intensity of red fluorescence significantly decreased with the addition of Nec-1, suggesting that necroptosis was partially suppressed. Our results indicate that the RIPK1/RIPK3/MLKL pathway mediates necroptosis in CRC cells treated with OSW-1. Additionally, OSW-1-induced necroptosis can be partially reversed by Nec-1, demonstrating protective effects in CRC cells. However, while the classical pathway is a crucial regulator of necroptosis, other mechanisms also contribute to this process[40-42]. Further investigations are warranted to elucidate whether different pathways and molecules are involved in OSW-1-triggered necroptosis in CRC cells.

According to our study, OSW-1-induced necroptosis activates RIPK1 and related signaling pathways. However, the specific mechanism by which OSW-1 activates RIPK1 has not been determined. To further elucidate the mechanism underlying RIPK1-dependent necroptosis in CRC, we investigated p62/SQSTM1, which is binds to RIPK1 and regulates the ubiquitin-proteasome system and lysosomal autophagy in various diseases. Recent research has highlighted a connection between tumorigenesis and the upregulation or inefficient degradation of p62/SQSTM1[43]. High levels of p62/SQSTM1 have been shown to inhibit the activity of the E3 Ligase RNF168, increasing the sensitivity of cancer cells to radiotherapy[44,45]. Consistent with these findings, our data also indicated an accumulation of the p62/SQSTM1 protein levels after OSW-1 treatment, suggesting defective autophagic degradation of p62/SQSTM1-bound substrates, contributing to cancer therapy.

Notably, our results demonstrated that treatment of CRC cells with OSW-1 could lead to the formation of a complex between RIPK1 and p62/SQSTM1. We propose that the accumulation of p62/SQSTM1 induced by OSW-1 facilitates the formation of necrosomes, triggering the activation of the RIPK1/RIPK3 pathway and ultimately leading to necroptotic cell death. This hypothesis was confirmed by the data showing that OSW-1 treatment induced the interaction of p62/SQSTM1 with RIPK1 in CRC cells. Moreover, knockdown of p62/SQSTM1 resulted in decreased expression of p-RIPK, p-RIPK3 and p-MLKL. It would be worthwhile to explore whether other pathways and molecules regulate OSW-1-induced necroptosis in CRC cells.


A
HT29
HCT116
Control
OSW-1 (1 μM)
p62
β-actin
62 kDa
42 kDa
LC3II
LC3I
14 kDa
16 kDa
Relative protein expression (/β-actin)
B
Relative mRNA expression (/β-actin)
p62
LC3
C
mCherry
GFP
Merge
D
IP
Input
IgG
Anti: RIPK1
IB:RIPK1
IB:p62
76 kDa
62 kDa
E1
Control
OSW-1
OSW-1 + Nec-1
Green
Red
Intensity (a.u.)
Distance (μm)

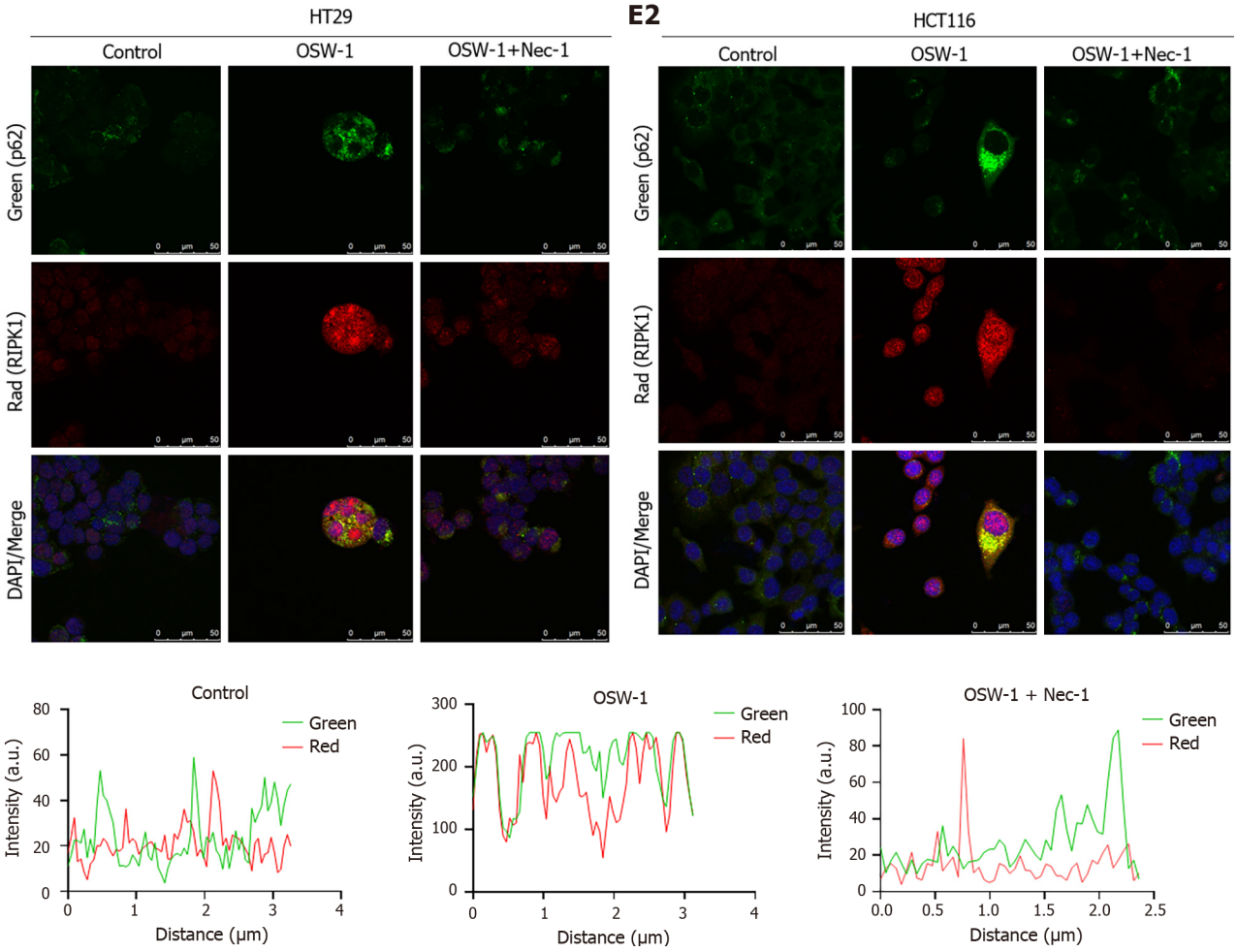


Figure 5 Impairment of the autophagic flux results in the accumulation of p62/SQSTM1, and p62/SQSTM1 can interact with RIPK1. A: The expression levels of p62/SQSTM1 and LC3II were evaluated in HT29 and HCT116 cells after exposure to OSW-1 for 24 h; B: The gene expression levels of p62/SQSTM1 and LC3 were assessed in HT29 and HCT116 cells after 24 h of exposure to OSW-1; C: Representative images of HT29 cells and HCT116 cells infected with adenovirus expressing GFP-mCherry-LC3. Cells not treated with OSW-1 served as the control. The images show total autophagosomes (yellow puncta) and functional autophagolysosomes (red puncta); D: Coimmunoprecipitation was conducted to assess the interaction between RIPK1 and p62/SQSTM1, and the results were analyzed through western blotting; E: Representative confocal microscopy images showing the colocalization of p62/SQSTM1 and RIPK1 in HT29 and HCT116 cells after exposure to OSW-1 for 24 h, with or without the addition of necrostatin-1. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* Control. Each data point represents the mean ± SE. PI: Propidium iodide; Nec-1: Necrostatin-1.

OSW-1-treated cells and nude mice exhibited typical necroptosis-like characteristics, as shown by increased expression of p-RIPK1, p-RIPK3, and p-MLKL. Moreover, p62/SQSTM1 plays a crucial role in the regulation of necroptosis. When OSW-1 causes intracellular damage, the accumulation of p62/SQSTM1 may act as a signaling platform to activate necroptosis, possibly through the inhibition of the autophagic flux. From a therapeutic perspective, our findings suggest that OSW-1 might induce necroptosis in cancer cells under conditions of deficient or defective autophagy. This discovery provides a rationale for further investigating the use of OSW-1 as a promising antitumor drug, particularly in the context of individualized therapeutic approaches.

CONCLUSION

Our results demonstrated that OSW-1 could induce necroptosis through the RIPK1/RIPK3/MLKL pathway, and this effect was potentially mediated by the RIPK1-p62/SQSTM1 complex. This study provides a novel mechanism on the antitumor effects of OSW-1 and may provide a new therapeutic target from a new perspective of CRC cell death, which has important clinical significance.

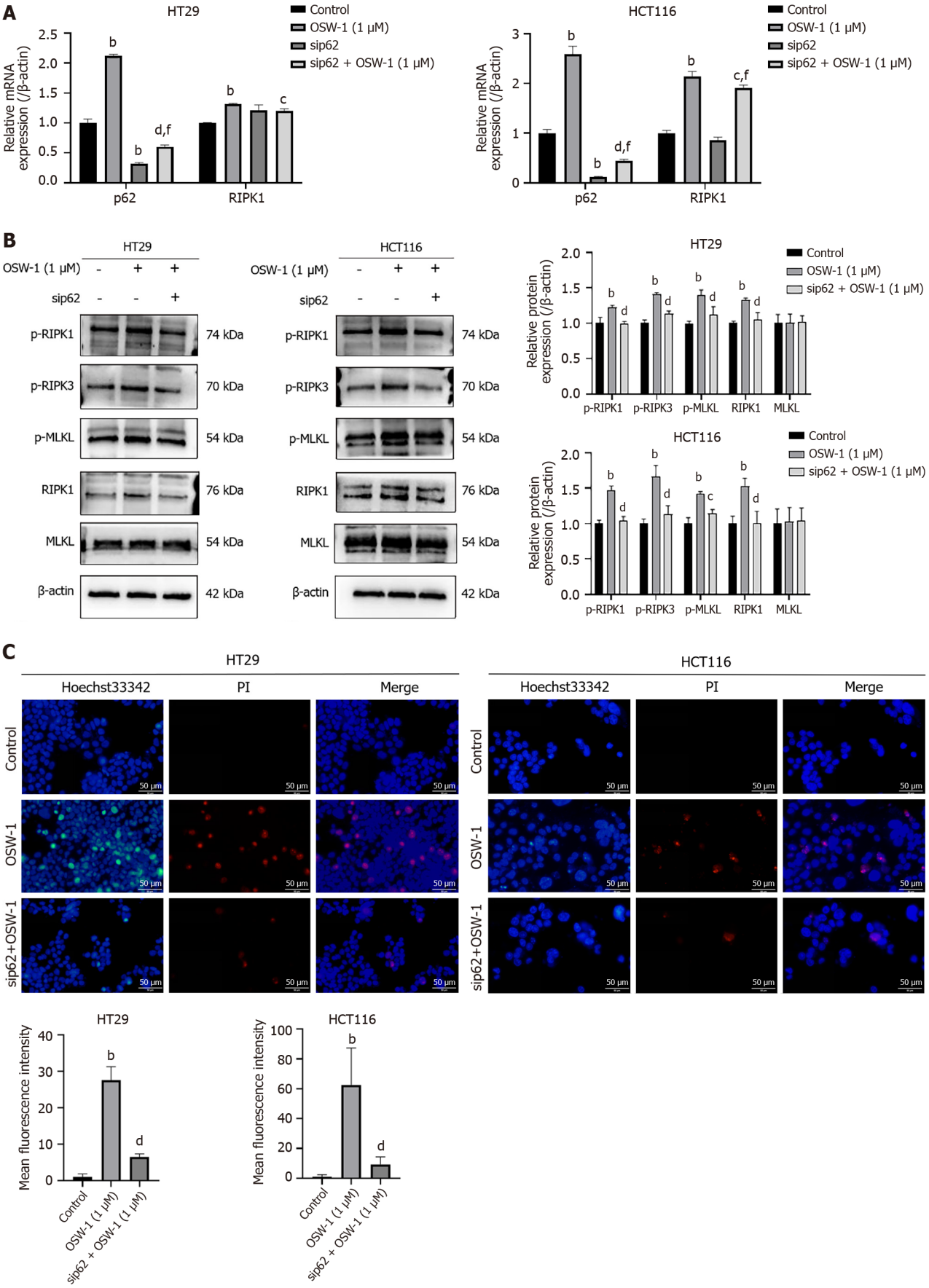


Figure 6 p62/SQSTM1 regulated OSW-1-induced necroptosis in colorectal cancer cells. A: The mRNA expression levels of p62/SQSTM1 and RIPK1 in HT29 and HCT116 cells were assessed after exposure to OSW-1 and p62/SQSTM1 siRNA; B: The protein expression levels of necroptosis-related proteins in HT29 and HCT116 cells were evaluated after treatment with OSW-1, with or without the addition of p62/SQSTM1 siRNA; C: A Hoechst 33342/propidium iodide dual

staining assay was used to assess necroptosis in HT29 and HCT116 cells following exposure to OSW-1, with or without the addition of p62/SQSTM1 siRNA. [b]*P* < 0.01 *vs* Control, [c]*P* < 0.05 and [d]*P* < 0.01 *vs* OSW-1, [f]*P* < 0.01 *vs* sip62. Each data point represents the mean ± SE. PI: Propidium iodide.

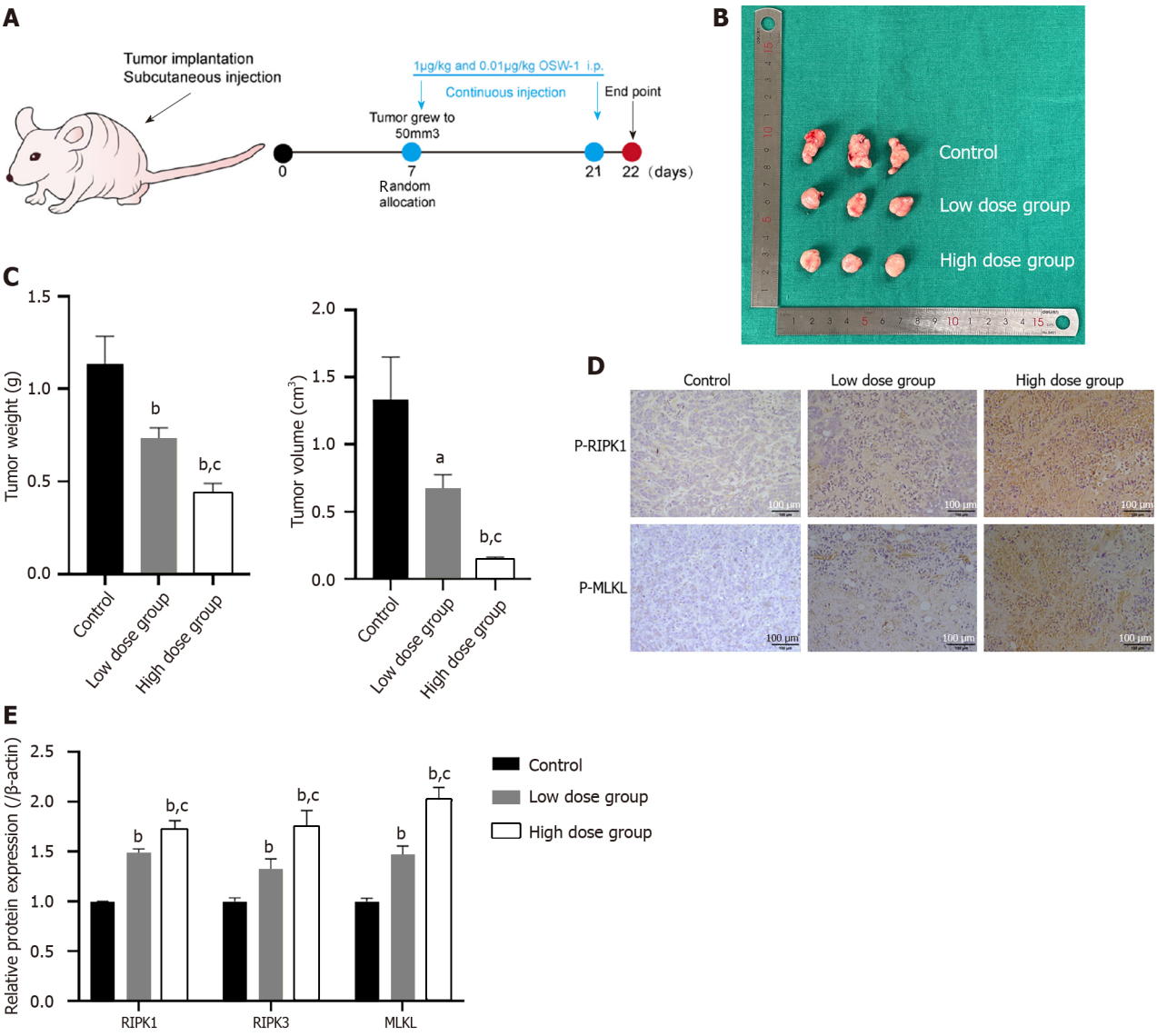


Figure 7 The inhibition of colorectal cancer cell proliferation is associated with necroptosis induced by OSW-1 *in vivo*. A: Treatment schedule for mice intravenously injected with 5 × 10^6 HT29 cells; B: Images of the harvested subcutaneous tumors. Tumor growth in the OSW-1 group was markedly suppressed compared to that in the control group; C: The tumor volume (mm^3) and weight in the high-dose OSW-1 group were lower than those in the low-dose group; D: Immunohistochemistry staining of p-RIPK1 and p-MLKL in xenograft nude mice (magnification, 200 ×); E: In xenograft nude mouse models, the expression levels of RIPK1, RIPK3 and MLKL in the high-dose group were increased in comparison to the low-dose group. [a]*P* < 0.05 and [b]*P* < 0.01 *vs* Control, [c]*P* < 0.05 and [d]*P* < 0.01 *vs* Low dose group.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) represents a significant health concern worldwide, and it has severe impacts on human lives. The search for effective drugs is crucial. OSW-1, which is derived from the bulbs of *Ornithogalum saundersiae*, exhibits potent antitumor properties. However, whether OSW-1 induces necroptosis in CRC cells to exert anticancer effects remains unclear.

Research motivation

We conducted a tandem mass tag proteomic analysis to elucidate the mechanisms underlying necroptosis. We explored the potential for the use of OSW-1 as a drug for the treatment of CRC.

Research objectives
This research aimed to investigate the influence of OSW-1 on CRC cells and elucidate the mechanisms underlying necroptosis.

Research methods
We performed a sequence of functional experiments, including Cell Counting Kit-8 assays and flow cytometry analysis, to assess the inhibitory impact of OSW-1 on CRC cells. We utilized quantitative proteomics to analyze changes in protein expression. transmission electron microscopy (TEM) and immunofluorescence studies were also performed to examine the effects of OSW-1 on necroptosis. Additionally, western blotting, siRNA experiments, and immunoprecipitation were employed to evaluate protein interactions within CRC cells.

Research results
The results revealed a pronounced inhibitory effect of OSW-1 on CRC cells, which was accompanied by a necroptosis-like morphology that was observed *via* TEM. OSW-1 was shown to trigger necroptosis *via* activation of the RIPK1/RIPK3/MLKL pathway. Furthermore, the accumulation of p62/SQSTM1 was shown to mediate OSW-1-induced necroptosis through its interaction with RIPK1.

Research conclusions
We propose that OSW-1 can induce necroptosis through the RIPK1/RIPK3/MLKL signaling pathway, which is facilitated by the RIPK1-p62/SQSTM1 complex in CRC cells.

Research perspectives
This study provides a novel mechanism on the antitumor effects of OSW-1 and may provide a new therapeutic target from a new perspective of CRC cell death, which has important clinical significance.

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FOOTNOTES

Co-first authors: Nan Wang and Chao-Yang Li.

Author contributions: Guo HS designed and supervised the study and drafted the manuscript; Wang N, Li CY, Yao TF and Kang XD performed the experiments; Yao TF and Kang XD analyzed the data; and Wang N and Li CY prepared the manuscript; All the authors have read and approved the final version of the manuscript. The reasons for designating Wang N and Li CY as co-first authors are twofold. Wang N and Li CY completed all the *in vitro* and *in vivo* experiments of this study, made the same contribution to this work and share the first authorship. Second, our study was a collaborative effort, and the co-first authorship accurately mirrors the shared responsibilities and collaborative work throughout the study and paper completion. This approach enhances effective communication and facilitates the management of post submission tasks, ultimately contributing to the improved quality and reliability of the paper. In summary, we firmly believe that designating Wang N and Li CY as co-first authors are fitting for our manuscript, as they faithfully represent our team's collaborative spirit, equal contributions, and diversity.

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LETTER TO THE EDITOR

Circulating tumor DNA in liquid biopsy: Current diagnostic limitation

Shi-Cai Liu

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Abstract

With the rapid development of science and technology, cell-free DNA (cfDNA) is rapidly becoming an important biomarker for tumor diagnosis, monitoring and prognosis, and this cfDNA-based liquid biopsy technology has great potential to become an important part of precision medicine. cfDNA is the total amount of free DNA in the systemic circulation, including DNA fragments derived from tumor cells and all other somatic cells. Tumor cells release fragments of DNA into the bloodstream, and this source of cfDNA is called circulating tumor DNA (ctDNA). cfDNA detection has become a major focus in the field of tumor research in recent years, which provides a new opportunity for non-invasive diagnosis and prognosis of cancer. In this paper, we discuss the limitations of the study on the origin and dynamics analysis of ctDNA, and how to solve these problems in the future. Although the future faces major challenges, it also contains great potential.

Key Words: Cell-free DNA; Circulating tumor DNA; Liquid biopsy; Cancer; Diagnosis; Prognosis

Core Tip: Tumor liquid biopsy based on cell-free DNA detection has become a major hotspot in the field of tumor research in recent years. Circulating tumor DNA (ctDNA) is a DNA fragment that breaks down from cells in primary tumors or even new tumors formed by metastasis, and enters the peripheral circulation. ctDNA analysis provides a non-invasive method for cancer detection and monitoring, which is important for the management of clinical patients.

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TO THE EDITOR

We read with interest a basic study by Terasawa *et al*[1], who assessed the origin of circulating tumor DNA (ctDNA), elucidated the dynamics of ctDNA levels, assessed ctDNA levels using xenografted mice after treatment, and determined whether tumor size and tumor invasion correlated with ctDNA levels. ctDNA is the cell-free DNA (cfDNA) of tumor origin. We congratulate the authors on their work and contributions in this field. At present, it is not clear what factors (*e.g.*, tumor size and tumor invasion) affect the levels of ctDNA, and there are always questions about the origin of ctDNA. It is crucial to address these issues in order to make ctDNA an effective and practical biomarker for liquid biopsy in clinical practice, and to fully tap into its potential. In this regard, Terasawa *et al*[1] explored the origin and dynamics of ctDNA with potential contributions to this issue.

Using BALB/c-nu/nu mice inoculated with the TE11 cell line, the authors established tumor xenotransplantation. In order to perform ctDNA analysis, several groups of mice were killed at appropriate time points after xenotransplantation. The findings shed light on the origin and dynamics of ctDNA, suggesting that tumor size is an important factor. Moreover, the results of this study showed that when the tumor was completely removed, the ctDNA disappeared after ≥ 1 d. Although the results were satisfactory, in future studies, researchers need to further understand the biological characteristics of ctDNA (such as ctDNA release and clearance mechanisms) and improve the sensitivity of ctDNA detection. The authors also pointed out in their discussion that, with respect to residual tumors, although all mice underwent pathological autopsies in this study, it was not possible to completely identify residual tumors, which is essentially a sensitivity issue of ctDNA detection.

In the early stage of cancer, the content of ctDNA in plasma is low, and the number of somatic mutations based on a certain mutation is even lower[2]. The detection and analysis of ctDNA brings many obstacles to clinical cancer detection, especially early detection. Previous research has shown that only when the ctDNA content in cfDNA is ≥ 10%, accurate information of tumor can be obtained[3]. However, with the exception of some patients with advanced tumors who have high amounts of ctDNA in plasma, the ctDNA content in most patients with tumors does not meet this standard[4]. This makes detecting ctDNA difficult, especially in the early stages of cancer. At present, increasing the depth of sequencing is mainly used to improve the sensitivity and accuracy of ctDNA detection, but increasing the depth of sequencing may bring false positive results, because cfDNA of non-tumor origin may also carry various tumor-associated mutations[5]. These problems have always limited the clinical application of ctDNA liquid biopsy.

Due to these shortcomings, in addition to detecting tumor mutations, the field of liquid cancer biopsy is actively exploring non-invasive detection methods based on other characteristics of plasma cfDNA, such as fragment size[6,7], methylation[8-11], end coordinates[12], and chromatin accessibility of cfDNA[13], which are currently being studied. Renaud *et al*[14] used fragment length characteristics of cfDNA to diagnose metastatic castration-resistant prostate cancer. Heeke *et al*[15] used cfDNA methylation data to classify small cell lung cancer (SCLC) and discovered SCLC subgroups based on DNA methylation data, which can serve as potential biomarkers to guide patient classification and clinical precision treatment. The United States Food and Drug Administration has approved the first blood-based colorectal cancer (CRC) screening product, *SEPT9* gene testing[16,17]. The study by Jiang *et al*[12] showed that cancer-related end coordinates in plasma cfDNA can be applied to liver cancer early detection. Using plasma cfDNA and protein markers, Cohen *et al*[18] developed CancerSEEK, which can detect eight common cancers of the lung, colorectum, liver, stomach, esophagus, pancreas, breast, or ovary. In my previous studies, cfDNA chromatin open state was used to distinguish patients with esophageal cancer from individuals without cancer[13]. Li *et al*[19] developed a multimodal epigenetic sequencing analysis (MESA) method based on cfDNA, MESA, for the detection of CRC, which can capture and integrate various epigenetic features in cfDNA, such as cfDNA methylation and nucleosome occupancy. These studies open up new ideas for cfDNA-based liquid biopsy in non-invasive diagnosis. Many studies have shown that the total level of cfDNA is correlated with tumor staging[20,21], suggesting that cfDNA has prognostic potential. In addition, cfDNA can serve as a real-time indicator of therapeutic efficacy, allowing for earlier observation of therapeutic effects than clinical trials, thanks to its short half-life[22-24].

At present, research on improving the sensitivity of ctDNA detection mainly focuses on *in vitro* sequencing and analysis, such as detecting multi somatic mutations and integrating DNA methylation or fragmentation patterns[25,26]. An inherent challenge faced by all these methods is the low amount of ctDNA in the collected blood samples, which limits sensitivity. Increasing the volume of the blood sample can improve sensitivity of the detection. However, for patients who are weak or ill, it is impractical. In addition, some have proposed methods that are closer to tumor sampling or increase tumor DNA loss. These methods also have certain limitations, such as requiring prior knowledge of tumor location, being limited to specific primary tumors, requiring invasive surgery, and being costly. Recently, a research team has developed two intravenous inducers to improve the recovery of ctDNA in blood collection, which can temporarily delay the clearance of cfDNA in the body[27]. Uptake by liver-resident macrophages and degradation by circulating nucleases are two natural mechanisms by which cfDNA is cleared. In this study, the authors developed two intravenous inducers that can act on these mechanisms and improve the recovery rate of ctDNA when used 1-2 h before blood withdrawal. Although this strategy can significantly improve the sensitivity of ctDNA detection in preclinical models, the

safety and tolerability of the formulation, as well as whether this effect can be translated into human patients, remain to be determined.

With the rapid development of science and technology, cfDNA is rapidly becoming an important biomarker for tumor diagnosis, monitoring and prognosis, and this cfDNA-based liquid biopsy technology has great potential to become an important tool of precision medicine. Despite its enormous potential, there is still a long way to go. Research on blood sample collection, cfDNA isolation, and Next-Generation Sequencing data analysis is currently limited and needs to be focused on in the future. In addition, the biological characteristics of ctDNA are also of great research value. Meanwhile, it is necessary to confirm the effectiveness and practicability of cfDNA as a diagnosis/prognosis marker to further promote the clinical application of cfDNA-based liquid biopsy.

FOOTNOTES

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