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REVIEW

Predictors and optimal management of tumor necrosis factor antagonist nonresponse in inflammatory bowel disease: A literature review

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Abstract

Tumor necrosis factor-α (TNF-α) antagonists, the first biologics approved for treating patients with inflammatory bowel disease (IBD), are effective for the induction and maintenance of remission and significantly improving prognosis. However, up to one-third of treated patients show primary nonresponse (PNR) to anti-TNF-α therapies, and 23%-50% of IBD patients experience loss of response (LOR) to these biologics during subsequent treatment. There is still no recognized predictor for evaluating the efficacy of anti-TNF drugs. This review summarizes the existing predictors of PNR and LOR to anti-TNF in IBD patients. Most predictors remain controversial, and only previous surgical history, disease manifestations, drug concentrations, antidrug antibodies, serum albumin, some biologic markers, and some genetic markers may be potentially predictive. In addition, we also discuss the next steps of treatment for patients with PNR or LOR to TNF antagonists. Therapeutic drug monitoring plays an important role in treatment selection. Dose escalation, combination therapy, switching to a different anti-TNF drug, or switching to a biologic with a different mechanism of action can be selected based on the concentration of the drug and/or antidrug antibodies.

Key Words: Predictor; Management; Tumor necrosis factor antagonist; Primary nonresponse; Secondary nonresponse; Inflammatory bowel disease

Core Tip: Tumor necrosis factor-α (TNF-α) antagonists play an essential role in the management of inflammatory bowel disease (IBD). However, a significant number of patients experience primary or secondary nonresponse to these drugs. Here, we summarize relevant predictors of anti-TNF nonresponse in IBD and discuss the next steps for treating patients with primary or secondary nonresponse to anti-TNF agents.

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INTRODUCTION

Inflammatory bowel disease (IBD), an immune-mediated inflammation of the gastrointestinal tract characterized by repeated remission and relapse, comprises Crohn's disease (CD) and ulcerative colitis (UC). Traditionally, IBD has been considered a disease of the Western world, but the newly industrialized countries of Asia, Africa, and South America are experiencing a rapid increase in incidence[1-3]; therefore, IBD has become a global disease[4,5].

IBD is a lifelong disease and is incurable. Currently, medical therapy for IBD mainly includes traditional therapeutics such as 5-aminosalicylates, thiopurines, and steroids, biologics such as antitumor necrosis factor (anti-TNF) therapy, vedolizumab and ustekinumab, and novel small-molecule drugs such as Janus kinase (JAK) inhibitors.

Anti-TNF therapies, the first biologics approved for the treatment of patients with IBD, are effective for the induction and maintenance of remission and significantly improve prognosis[6-8]. The development of anti-TNF therapies revolutionized the treatment of IBD and was a landmark event. Anti-TNF drugs are still the most commonly used biological agents in IBD at present[6]. Four TNF antagonists have been used in the treatment of IBD: infliximab, adalimumab, certolizumab, and golimumab[9]. However, up to one-third of treated patients show no primary response to anti-TNF-α therapies[10], and 23%-50% of IBD patients experience loss of response (LOR) to these biologics during subsequent treatment[11,12]. These patients not only fail to benefit from anti-TNF therapies but also suffer from the side effects of anti-TNF drugs, including increased susceptibility to infection, autoimmune diseases, and malignant tumors[13,14]. In addition, they face a serious financial burden. A retrospective study reported that direct healthcare expenditures increased significantly after the initiation of anti-TNF therapy and remained higher than preinitiation costs for up to 5 years[15].

Hence, it is important to assess the therapeutic response to anti-TNF agents in IBD before initiating treatment. In this review, we conducted a comprehensive search of studies to summarize relevant predictors of anti-TNF nonresponse in IBD and discuss the next steps of treatment for patients with primary or secondary nonresponse (SNR) to anti-TNF agents.

LITERATURE SEARCH STRATEGY

We conducted a search on PubMed and Web of Science. Keywords used include “inflammatory bowel disease”, “Crohn's disease”, “Ulcerative colitis”, “Tumor necrosis factor antagonists”, “anti-TNF”, “infliximab”, “adalimumab”, “certolizumab”, “golimumab”, “primary nonresponse”, “secondary nonresponse”, and “loss of response”. This review included articles, reviews and guidelines that investigated predictors of failure of TNF antagonists in IBD or optimized treatment (Supplementary Figure 1).

DEFINITION

Primary nonresponse

There is no consensus on the definition of primary nonresponse (PNR) in IBD patients as definitions vary across studies. Papamichael *et al*[11] defined PNR as a lack of objectively assessed improvement in baseline inflammatory signs after induction treatment in the presence of adequate concentrations of the drug and in the absence of antidrug antibodies (ADAs). In a cohort study, PNR was classified as treatment failure or use of corticosteroids (new prescription or previous dose not discontinued) or failure to reduce C-reactive protein (CRP) to 3 mg/L or less or to decrease by 50% or more from baseline and failure to decrease Harvey-Bradshaw Index score to 4 or less or by 3 or more from baseline before week 14 [16]. In general, PNR refers to the absence of improvement in clinical symptoms or objective measures during the induction phase[17-19]. The incidence of PNR has been reported to range from 13%-40%[7,20,21].

Secondary nonresponse

SNR, also named LOR, describes the clinical phenomenon of patients who have an initial response to biologics but then subsequently lose this response[22]. Notably, the two features of the SNR are that the patient's symptoms improved

during the initial treatment and that the recurrence of symptoms can only be due to the inflammatory response of IBD and not due to concurrent infection, fibrous stenosis, *etc*.[23]. SNR eventually occurs in 20%-50% of patients[12,24,25]. A recent meta-analysis found that the mean percentages of patients with SNR to infliximab, adalimumab, and certolizumab were 37.8%, 35.4%, and 43.3%, respectively[26].

PREDICTORS OF PNR

Clinical features

Age: Real-world data suggest that elderly individuals with CD benefit less from infliximab and adalimumab at 12 wk [27]. In the precision-3 study, CD patients treated with certolizumab had a reduced probability of achieving a primary response as they aged[28]. However, several other studies have reported no correlation between age and PNR to anti-TNF in CD[21,29]. In UC patients, Arias *et al*[30] found that the benefit was greater when the baseline age was less than 40 years, whereas other studies did not show the impact of age on the efficacy[31,32]. Differences between the results may have originated from variations in designs and how outcomes were defined.

Gender: A single-center study in Britain involving CD patients reported that men were significantly less likely to PNR to infliximab[21]. Another Korean study showed that among CD patients, men benefited from clinical remission at week 14 more than women[29]. However, many researchers have not found an association between sex and PNR to anti-TNF therapy in CD[33-35]. Similarly, the influence of gender on anti-TNF therapy cannot be clearly defined in UC patients. Sandborn *et al*[36] reported that women responded better when assessing the efficacy of golimumab at week 6. Other studies did not report that sex could predict TNF antagonists response in UC[30,37].

Smoking: Smoking is an environmental risk factor for CD[38] and appears to be associated with nonresponse to anti-TNF therapy in CD patients. Analysis from the precision-3 study suggested that nonsmokers are more likely to achieve early clinical remission than smokers[28]. Zorzi *et al*[39] identified a positive association between smoking and anti-TNF nonresponse in CD patients by Cox proportional hazards regression. In addition, a meta-analysis published in 2021 revealed that when smoking status was defined smoking was significantly associated with a reduction in response to infliximab or adalimumab in patients with CD[40]. However, the negative effect of smoking on response was not found in another earlier meta-analysis[41]. Studies of UC have also reported inconsistent results. One Italian study found a significantly lower response to infliximab in ex-smokers[42], while others did not reach this conclusion[37,43]. The conflicting findings may be due to different definitions of smoking among the studies. In summary, smoking cessation is recommended for current smokers diagnosed with IBD[44].

Previous surgery: Although treatment strategies for IBD have changed, 17.4%-25% of patients with CD still require surgery[45-47]. Macaluso *et al*[27] used a logistic regression model to identify a history of previous surgery as an independent risk factor for PNR in CD patients. Another group reported similar results, showing that CD patients without previous surgery had a greater chance of achieving initial remission than patients with previous surgery, with a hazard ratio of 1.387[28]. CD patients with previous surgery had a lower response rate[48]. A study involving 201 CD patients also demonstrated that previous surgery was an independent predictor of PNR[34].

Disease duration: The analysis of pooled data from CD studies indicates that CD with a shorter disease duration is associated with a superior early response[49]. In the MODIFY study, patients who received early adalimumab achieved a higher clinical response and remission rate at week 26 than those who received delayed treatment[50]. This correlation has also been confirmed by a recent meta-analysis[51]. Studies have reported that among UC patients, a shorter disease duration is associated with a better response to anti-TNF drugs[32,52]. However, in general, authors did not find a positive association between long disease duration and anti-TNF nonresponse[31,48,53]. Although the current studies available cannot explain the underlying reasons for poorer response to anti-TNF in IBD with a longer disease duration, it is intuitive that a longer disease duration may contribute to the development of fibrosis, making earlier treatment attractive to patients[54].

Phenotype: The disease phenotype seems to be related to anti-TNF treatment response. In CD patients, isolated ileitis was inversely associated with the anti-TNF response, whereas the opposite was true for isolated colitis[29,48].

Pharmacokinetic

The pharmacokinetic (PK) of anti-TNF consists of four processes: absorption, distribution, metabolism, and elimination [55]. PK failures are characterized by undetectable or subtherapeutic drug concentrations associated with rapid nonimmune clearance or immunogenicity as well as the development of ADAs[56].

Drug concentration and antidrug antibodies: Several studies have demonstrated that subtherapeutic drug concentration is a predictor of PNR, with drug concentrations lower in IBD patients who failed to respond to anti-TNF therapy than in responders[16,57]. Post hoc analysis of data from the MUSIC trial data showed that CD patients with higher levels of certolizumab were more likely to achieve endoscopic response and remission at week 10[58]. Ding *et al*[17] suggested that low anti-TNF levels and the formation of ADAs could predict PNR in CD patients. The same results were reported in another study involving patients with UC[59].

Weight: Weight is a predictor of anti-TNF nonresponse. In a multicenter cohort study, high body mass index (BMI) at baseline in CD patients was associated with an increased risk of PNR[16]. Similar results were reported in another study [60]. In UC patients, Kurnool *et al*[61] reported that an increase in BMI had a negative impact on the response to anti-TNF drug therapy. The reason may be that, on the one hand, obesity induces a proinflammatory state[62], and on the other hand, the proteolytic clearance of immunoglobulins is usually related to weight, that is, the higher the weight is, the faster the clearance[63,64].

Serum albumin: Serum albumin levels predict the PK of anti-TNF therapy. A recent prospective study noted that low albumin levels at baseline in IBD patients predicted low infliximab concentrations at week 14[16]. Several other studies have reached similar conclusions[57,63]. One study of patients with UC found significantly higher serum albumin in responders than in primary nonresponders[65]. This effect occurs because albumin is the main transporter of drugs in blood, and serum albumin binds anti-TNF drugs to protect against degradation[66].

Fcγ receptor type IIIA: Single nucleotide substitutions within the Fcγ receptor type IIIA (FCGR3A) gene result in allelic variations, one valine (V) or one phenylalanine (F) at amino acid position 158. Functional polymorphisms in FCGR3A are significantly associated with response to anti-TNF therapy in CD patients[67]. Bek *et al*[67] used mono-compartmental population modeling to describe the PK of infliximab and found that the FCGR3A-158V/V genotype was associated with increased elimination of infliximab[67]. Further studies identified the FCGR3A VV phenotype as an independent predictor of ADAs generation and associated with a reduced clinical response in IBD patients at the end of induction[68].

Pharmacodynamic

Pharmacodynamic (PD) failure is associated with underlying non-TNF-driven inflammation characterized by no improvement in symptoms even at sufficient concentrations and without ADAs[56].

Pharmacokinetic/pharmacodynamic modeling: Kimura *et al*[69] developed Pharmacokinetic/pharmacodynamic (PK/PD) modeling to predict IBD response to infliximab during induction therapy. Another team of researchers in Japan developed a PK/PD model to calculate the $K^{anti\text{-}TNF\alpha}{}_0/K^{else}$ ratio to predict the PNR to TNF in IBD patients at the second administration[70]. The validity of this model remains to be tested in larger populations.

Biologic markers

C-reactive protein: Several studies have investigated the relationship between CRP levels and anti-TNF responses. A multicenter retrospective study in Korea demonstrated that UC patients with CRP ≥ 3 mg/dL were more likely to achieve clinical remission at week 8[71]. This was also observed in CD treated with certolizumab[72]. However, opposite results were reported in another retrospective study of CD patients[73]. Presumably, high baseline CRP will exclude some patients with noninflammatory functional symptoms and predict a higher response, but it may also reflect a higher inflammatory load with increased drug loss[74].

Antineutrophil cytoplasmic antibody and anti-*Saccharomyces cerevisiae* antibody: In a cohort study involving 90 UC patients, a greater proportion of antineutrophil cytoplasmic antibody (ANCA)-negative patients achieved clinical response during infliximab induction than ANCA-positive patients[75]. Another study in CD patients found that positive perinuclear ANCN (pANCA) is a predictor of anti-TNF nonresponse[76]. In a meta-analysis, pooled results showed that pANCA-negative patients with IBD had a nearly twofold higher response to anti-TNF therapy than pANCA -positive patients[77]. A single-center study evaluating pANCA and anti-*Saccharomyces cerevisiae* antibody (ASCA) simultaneously found that pANCA+/ASCA- serotypes significantly reduced early clinical response to infliximab in CD patients[78].

Fecal calprotectin: Fecal calprotectin (FC) is an indicator of gut inflammation and disease burden in IBD. Beltrán *et al*[79] noted that FC was higher in PNR patients with CD than in responders at weeks 0, 6, and 14, with a statistically significant difference only at week 0. Another study in UC patients also showed that early high FC was predictive of infliximab nonresponse[52]. Pavlidis *et al*[80] suggested that a decrease in FC of less than 70% after induction with anti-TNF drugs could predict PNR in patients with CD. However, some authors have not shown a relationship between FC and anti-TNF PNR in UC patients[81,82].

Fecal lactoferrin: Fecal lactoferrin (FL) can be used to monitor intestinal inflammation in IBD[83]. A retrospective study involving IBD demonstrated that dynamic monitoring of FL could distinguish responders from primary nonresponders, with two sustained drops in FL observed in responders during induction therapy[84].

Genetic markers

TNF and TNF-receptor superfamily genes: Genetic polymorphisms associated with TNF and TNF receptors have been widely studied for their ability to predict the response to anti-TNF therapy. In a clinical trial studying CD, patients homozygous for the TNF/Lymphotoxin alpha (LTA) polymorphism, the LTA NcoI-TNFc-aa13L-aa26 haplotype 1-1-1-1, showed early nonresponse to infliximab[76]. Another study demonstrated that TNF-308 (rs1800629) was associated with response to anti-TNF therapy, and the presence of the minor allele (A) was associated with increased odds of nonresponse to anti-TNF therapy in IBD[85]. For TNF-receptor superfamilies (TNFRSF), Steenholdt *et al*[86] found that CD patients carrying the TNFRSF1B minor allele rs1061622 had a better response to infliximab induction therapy. In a Japanese study, TNFRSF1A (rs767455_G) and TNFRSF1B (rs1061624_A-rs3397_T) were associated with poor response in CD patients[87] and these results were replicated in another Spanish study[88]. Additionally, a meta-analysis indicated

that TNFRSF1A (rs4149570) significantly improved anti-TNF responses in IBD[67].

Autophagy-related 16 like 1: Autophagy-related 16 like 1 (ATG16L1) is a risk factor for CD[89]. Koder *et al*[90] found a strong association between ATG16L1 (rs10210302) and response to adalimumab treatment in the CD population, with the TT genotype showing a better response after 12 wk of adalimumab treatment. Future studies on the relationship between ATG16L1 and the anti-TNF response are necessary to clarify these effects.

Apoptosis genes: Infliximab and adalimumab induce apoptosis by binding to membrane-bound TNF-α, which is the main mechanism of their efficacy[54]. An earlier study observed the strongest association between the Fas ligand -843 TT genotype and nonresponse to infliximab in CD patients[91]. Furthermore, Hlavaty *et al*[92] developed a novel apoptotic pharmacogenetic index based on three single nucleotide polymorphisms (Fas ligand-843 C/T, Fas-670 G/A, and Caspase9 93 C/T), with a higher score indicating a better response to anti-TNF therapy.

Nucleotide-binding oligomerization domain 2: Nucleotide-binding oligomerization domain 2 (NOD2) mutations predict an increased risk of complications related to CD[93]. Further studies showed that NOD2 mutations were less responsive to anti-TNF therapy than wild-type NOD2 in CD patients[94]. Another study demonstrated that CD patients with either NOD2 variants alone or in combination with ATG16L1 variants were more likely to receive intensive biologic therapy, which may indicate that NOD2 variants have a negative impact on response to biologic therapy[95]. However, this effect was not observed in another trial[96].

Cytokines

Interleukin: One study conducted in CD patients found that primary nonresponders had significantly higher interleukin-8 (IL-8) concentrations at baseline[97]. In addition, the level of IL-6 in responders was significantly lower than that in primary nonresponders at week 2 and week 6[97]. Another study of CD noted that IL17A and IL1B expression was significantly upregulated in anti-TNF refractory patients during anti-TNF therapy[98]. Oncostatin M (OSM), a member of the IL-6 cytokine family, has been shown to disrupt epithelial barrier function and drive intestinal inflammation[99]. An analysis of more than 200 IBD patients treated with anti-TNF therapy found higher baseline levels of OSM expression in those who failed anti-TNF therapy[100]. A cross-sectional study suggested that higher levels of OSM in the colon were associated with PNR to anti-TNF in patients with IBD[101].

Triggering receptor expressed on myeloid cells 1: Triggering receptor expressed on myeloid cells 1 (TREM1) expression has been proposed as a potential marker for predicting response to anti-TNF therapy in IBD patients. Gaujoux *et al*[102] demonstrated that TREM1 can be an ex-ante predictor of the anti-TNF response and that TREM1 Levels were downregulated in nonresponders, with a prediction accuracy of 94%. This phenomenon is also found in the inflamed mucosa.

Gut microbes

Several studies have shown that gut microbes predict nonresponse to anti-TNF treatment in IBD. Magnusson *et al*[103] found that responders had lower dysbiosis indexes, a higher abundance of *Faecalibacterium prausnitzii* (*F. prausnitzii*) at baseline, and an increase in the abundance of *F. prausnitzii* during induction therapy compared with nonresponders. Another study found that high abundances of the genera *Blautia*, *Faecalibacterium*, *Roseburia*, and *Negativibacillus* at baseline were inversely associated with responsiveness to infliximab in CD[104]. In the same study, a high abundance of *Sutterella*, *Roseburia*, and *Intenstinibacter* appeared to predict response to infliximab in UC patients[104]. Alatawi *et al*[105] detected a reduction in the abundance of short-chain fatty acid-producing bacteria, including *Anaerostipes*, *Coprococcus*, *Lachnospira*, *Roseburia*, and *Ruminococcus,* in IBD patients unresponsive to anti-TNF therapy[105]. Nevertheless, a European multicenter study found no differences in the microbiota of anti-TNF therapy responders *vs* nonresponders in IBD[106]. Indicators that predict PNR to anti-TNF agents in patients with IBD are listed in Table 1.

PREDICTORS OF SECONDARY NONRESPONSE

Clinical features

Gender: A retrospective study identified that women were more likely to develop SNR to anti-TNF[107]. Another multicenter retrospective study found a similar result in the CD subgroup[108]. An earlier systematic review noted the male sex was a predictor of LOR in CD[109]. A single-center study demonstrated a significantly higher likelihood of SNR in men with UC[110]. However, no association has been reported between gender and SNR in most studies[21,30,31,37].

Smoking: Sandborn *et al*[28] found that current smoking was associated with LOR in individuals diagnosed with CD. This result was also validated in another single-center study[39]. Chaparro *et al*[111] reported that smoking was associated with the occurrence of LOR in CD.

Previous surgery: A Sicilian study of CD reported that previous surgery was associated with a low rate of clinical remission at 1 year[27]. However, many studies have not demonstrated a relationship between previous surgical history and SNR to anti-TNF therapy in CD[16,112].

Disease duration: Panaccione *et al*[49] showed that patients with CD whose duration was less than 1 year benefited more in maintaining remission. A retrospective cohort study reported that CD patients with a disease duration of more than 2 years had a significantly higher rate of SNR[113]. A subgroup analysis of the placebo-controlled CHARM trial also

Table 1 Predictors of primary nonresponse in Crohn's disease and ulcerative colitis

Predictor	Crohn's disease	Ulcerative colitis
Clinical features		
Age	Yes: Older[27,28]; No[21,29]	Yes: Older[30]; No[31,32]
Gender	Yes: Male[21], female[29]; No[33-35]	Yes: Male[36]; No[30,37]
Smoking	Yes: Smoker[28,39,40]; No[41]	Yes: Ex-smoker[42]; No[37,43]
Previous surgery	Yes[27,28,34,48]	
Disease duration	Yes: Longer[49-51]; No[48]	Yes: Longer[32,52]; No[30,53]
Phenotype	Yes: Isolated ileitis[29,48]	
Pharmacokinetic		
Drug concentration	Yes: Low[16,17,57,58]	Yes: Low[57,59]
Antidrug antibodies	Yes[17]	Yes[59]
Weight	Yes: High[16,60]	Yes: High[61]
Serum albumin	Yes: Low[57]	Yes: Low[57,65]
FCGR3A	Yes: FCGR3A-158V/V[67,68]	Yes: FCGR3A-158V/V[68]
Pharmacodynamic		
PK/PD model	Yes[69,70]	Yes[69,70]
Biologic markers		
CRP	Yes: Low[72], High[73]	Yes: Low[71]
ANCA and ASCA	Yes: pANCA+[76,77]	Yes: ANCA+[75], pANCA+[77], pANCA+/ASCA-[78]
Fecal calprotectin	Yes: High[79]	Yes: High[52]; No[81,82]
Fecal lactoferrin	Yes: High[84]	Yes: High[84]
Genetic markers		
TNF genes	Yes: Lymphotoxin alpha NcoI-TNFc-aa13L-aa26 haplotype 1-1-1-1[76], TNF-308A[85]	Yes: TNF-308A[85]
TNFRSF	Yes: TNFRSF1A (rs767455_G)[87], TNFRSF1B (rs1061624_A-rs3397_T)[87]	
ATG16L1	Yes: ATG16L1 (rs10210302_CC)[90]	
Apoptosis genes	Yes: Fas ligand-843 TT genotype[91]	
NOD2	Yes: NOD2 mutation[94,95]	
Cytokines		
Interleukin	Yes: IL-8 (high)[97], IL-6 (low)[97], IL17A (high)[98], IL1B (high)[98], OSM (high)[100,101]	Yes: OSM (high)[100,101]
TREM1	Yes: Low[102]	Yes: Low[102]
Gut microbes	Yes: Abundance of short-chain fatty acid-producing bacteria (decreased)[105]	Yes: Dysbiosis indexes (high)[103]; Abundance of short-chain fatty acid-producing bacteria(decreased)[105]

FCGR3A: Fcγ receptor type IIIA; PK/PD model: Pharmacokinetic/pharmacodynamic modeling; CRP: C-reactive protein; ANCA: Antineutrophil cytoplasmic antibody; ASCA: Anti-*Saccharomyces cerevisiae* antibody; pANCA: Perinuclear anti-neutrophil cytoplasmic antibody; TNF: Tumor necrosis factor; TNFRSF: TNF-receptor superfamily; ATG16L1: Autophagy-related 16 like 1; NOD2: Nucleotide-binding oligomerization domain 2; OSM: Oncostatin M; TREM1: Triggering receptor expressed on myeloid cells 1.

obtained a similar conclusion[114].

Phenotype: A recent study reported that accumulation of the upper digestive tract and the presence of fistulas at baseline were associated with SNR to adalimumab and infliximab in CD patients[27]. Another study involving 93 individuals verified that nonstructuring nonpenetrating CD was associated with sustained remission[39]. CD with concurrent fistula or stenosis had a lower clinical remission rate[115].

Pharmacokinetic

Drug concentration and antidrug antibodies: A multicenter cohort study confirmed that concentrations of infliximab < 7 mg/L and adalimumab < 12 mg/L were independently associated with SNR in CD patients[16]. A prospective study indicated that the trough level (TL) of infliximab < 5.5 μg/mL in patients with IBD was the best threshold to predict LOR [116]. Alternatively, the generation of ADAs, which in combination with circulating drugs also leads to a reduction in drug concentration, is associated with anti-TNF LOR in IBD[117].

Weight: Kennedy *et al*[16] found that obesity at baseline was associated with adalimumab treatment failure at week 54 in patients with CD. Another study also reported that IBD patients with a high BMI displayed a high rate of LOR[118]. In IBD patients treated with adalimumab, SNR was increased in those with BMI ≥ 30 kg/m^2 compared with those with BMI < 30 kg/m^2[119].

Serum albumin: In CD patients treated with certolizumab, low albumin predicted SNR[28]. Higher albumin levels were associated with lower LOR in IBD patients treated with infliximab[119]. A prospective study found that IBD patients with low albumin serum concentrations at baseline had a significantly increased risk for SNR to anti-TNF and that normalization of albumin levels during treatment did not reduce this risk[120].

Serum γ-globulin: A German study from IBD found a positive association between elevated serum γ-globulin concentrations and the risk of SNR to anti-TNF therapy[120]. Higher γ-globulin concentrations imply increased B-cell activity, resulting in increased ADAs production[120].

Matrix metalloproteinase 3: Matrix metalloproteinase 3 (MMP3) expression is significantly upregulated in inflamed colonic segments of IBD patients, suggesting the possible involvement of this enzyme in the inflammatory process[121, 122]. A retrospective study from Italy showed that in IBD patients, MMP3 levels were significantly lower in responders (11.48 ng/mL) than in nonresponders (25.96 ng/mL) at week 52[123]. In the same study, MMP3 levels tended to be higher in patients without ADAs than in those with ADAs[123]. According to a previous report, MMP3 cleaved infliximab and adalimumab which may result in reduced drug efficacy[124].

Fcγ receptor type IIIA: A Spanish team found higher serum concentration levels of both infliximab and adalimumab in FCGR3A FF carriers than in FCGR3A VV carriers during maintenance therapy in IBD and found that the proportion of VV patients who developed ADAs was significantly higher than that of FF patients diagnosed with IBD[125].

Human leukocyte antigen: The value of human leukocyte antigen-DQA1*05 (HLA-DQA1*05) in predicting anti-TNF-ADAs production has been reported in several studies. A genome-wide analysis of 1240 subjects in the PANTS cohort revealed that approximately 40% of Europeans carried HLA-DQA1*05 and significantly increased rates of ADAs production[126]. Wilson *et al*[127], using genotypic analysis, showed that HLADQA1*05 was independently associated with LOR to infliximab and increased ADAs in IBD.

Biologic markers

C-reactive protein: Post hoc analysis of ACCENT I, indicated that high levels of CRP before treatment predicted an increased likelihood of maintaining remission[128]. A study of IBD found that CRP > 5 mg/L was an independent predictor of SNR[116]. However, a Hungarian study reported that low levels of CRP at week 12 were associated with clinical remission at week 52 in CD patients on adalimumab[129]. Additionally, Angelison *et al*[82] did not find an association between CRP and SNR to anti-TNF agents in UC.

Antinuclear antibody: Among patients with IBD, those with positive antinuclear antibody (ANA) at baseline had higher odds of LOR to anti-TNF[130]. More studies are needed to investigate the relationship between ANA and response to anti-TNF therapy in the future.

Fecal calprotectin: Analyses from the 7-year PRECiSE 3 study revealed that an increase in FC implies an increased risk of LOR to anti-TNF[28]. However, Deshpande *et al*[131] reported that FC levels at week 14 could not predict the recurrence of CD one year later. Differences in the timing of FC measurement and sample size may have contributed to this discrepancy.

Fecal lactoferrin: Sorrentino *et al*[132] found that FL levels before and after anti-TNF treatment could be used to distinguish responders, partial responders, and nonresponders in IBD patients with suspected LOR[132]. In the same study, they proposed that responders had normal FL both before and after administration, partial responders had elevated FL before administration, partial FL decreased after administration but remained well above the normal threshold, and FL increased after LOR administration[132].

Genetic markers

TNF and TNF-receptor superfamily genes: Currently, only a retrospective cohort study of CD has demonstrated that carrying the TNFRSF1B minor allele rs976881 was associated with LOR to infliximab[86]. More studies are urgently needed to explore the relationship between TNF and TNFRSF genes and SNR to anti-TNF therapy.

Cytokines

Interleukin: Higher baseline OSM in IBD patients with SNR to infliximab was found in a UK study[100]. Bertani *et al*[133]

demonstrated that in CD patients treated with infliximab, those with low OSM levels at baseline and week 14 were more likely to achieve clinical remission at week 54[133]. Moreover, the level of OSM in patients with mucosal healing was significantly lower than that in patients without mucosal healing at week 54[133]. We summarize the predictors of SNR in Table 2.

OPTIMAL MANAGEMENT OF ANTI-TNF NONRESPONSE

Assessment

PNR or SNR to anti-TNF therapy was determined according to clinical symptoms, laboratory tests, endoscopy, imaging examinations, *etc.* It is worth noting that conditions such as poor adherence[134], improper drug storage medication storage[135], and co-infection[23] need to be excluded during assessment.

Therapeutic drug monitoring

The British Society of Gastroenterology consensus defines therapeutic drug monitoring (TDM) as, the measurement of the drug (± ADAs) levels to assess compliance, drug metabolism, and immunogenicity with a view to guide dose adjustments or switch off therapy[136]. TDM can be used reactively or proactively. The American Gastroenterological Association recommends reactive TDM for adults who fail to respond to anti-TNF therapy[9]. A target TL of at least 5 μg/mL, 7.5 μg/mL, and 20.0 μg/mL for infliximab, adalimumab, and certolizumab, respectively, is suggested[9]. Papamichael *et al*[137] recommend a minimum drug concentration of at least 2.5 μg/mL at week 6 and a trough concentration of at least 1 μg/mL of golimumab during maintenance therapy. Several recent reviews showed that TDM was more beneficial than empirical strategies in terms of cost-effectiveness[138-140]. TDM plays an important role in optimizing anti-TNF therapy.

Management of PNR

There is no consensus on the optimal management of PNR to TNF antagonists. A review proposed that the management of IBD patients with PNR to anti-TNF therapy consists of three major steps: prediction, prevention, and therapeutic intervention[11]. Clinical features, pharmacokinetics, genetic phenotypes, *etc*., can predict the development of PNR. Preventive measures to avoid PNR to anti-TNF include counseling patients to quit smoking, weight intervention, *etc*.[11, 17]. For IBD patients with PNR, empirical intervention can be performed, switching to another TNF antagonist, or switching to a biological agent of a different mechanism, is desirable[141]. Ding *et al*[17] suggested that a second TNF antagonist be administered when the patient is PNR to the first TNF antagonist. If the treatment fails again, switching out of class should be considered.

Some scholars have also proposed that the medication of primary nonresponders can be adjusted according to TDM. With the help of TDM, rational and optimal treatment can be provided[136]. If patients have low TLs and no or low titer ADAs formation, dose optimization or the addition of an immunomodulator is recommended. When TLs are low and high-titer ADAs are detected, switching to another TNF antagonist or biologic agent with a different mechanism may be considered. For patients with therapeutic concentrations, switching out of class is suggested (Figure 1).

Management of secondary nonresponse

The detection of TNF antagonist and ADAs concentration is helpful to guide the next treatment of SNR (Figure 2).

Dose escalation: Dose intensification can reverse nonresponse to anti-TNF in IBD patients with subtherapeutic concentrations and no or low concentrations of ADAs. A meta-analysis reported a 34% need for anti-TNF dose escalation in CD at a median follow-up of 1 year, with pooled rates of 38%, 32%, and 2% for infliximab, adalimumab, and certolizumab, respectively[26]. A multicenter cohort study in Belgium found that 34% of CD patients treated with adalimumab required an increased dose to maintain clinical response, and clinical response was induced again in 67% of these patients[142]. Billioud *et al*[109] concluded that among CD patients who experienced LOR to adalimumab, 71.4% regained response and 39.9% achieved remission after dose optimization. Interestingly, a post hoc analysis of the TAXIT trial showed a significantly higher rate of clinical response with dose intensification, regardless of the presence of ADAs[143]. Meanwhile, Bodini *et al*[144] have suggested that, based on clinical need, anti-TNF doses can be increased, even in older patients of patients receiving combination therapy, with little risk of adverse reactions occurring.

Addition of an immunomodulator: The addition of an immunomodulator is a good option for IBD patients receiving anti-TNF therapy in whom subtherapeutic and no or low concentrations of ADAs are detected. For example, van Schaik *et al*[145] observed a significant increase in mean trough concentrations and a significant decrease in the incidence of ADAs in the infliximab combined with azathioprine group compared with infliximab alone in patients with IBD, whereas no differences were observed in the adalimumab combination *vs* monotherapy groups[145]. Another study involving patients with CD reported that, for both infliximab and adalimumab, combined immunomodulators reduced the risk of ADAs formation[16]. In the SONIC trial, the response rate in corticosteroid-free clinical remission at week 50 was significantly higher with infliximab adding immunomodulator than with monotherapy (55.6% *vs* 39.6%)[146]. In the UC-SUCCESS trial, infliximab plus an immunomodulator was also superior in achieving corticosteroid-free clinical remission [147]. In a 2-year cohort study of 46 patients with IBD, the addition of a low-dose immunomodulator, either azathioprine, methotrexate, or mycophenolate mofetil, reversed clinical response in approximately 50% of IBD patients who had failed to respond to anti-TNF monotherapy[148]. With regard to when to discontinue immunomodulators, Drobne *et al*[149]

Table 2 Predictors of secondary nonresponse in Crohn's disease and ulcerative colitis

Predictor	Crohn's disease	Ulcerative colitis
Clinical features		
Gender	Yes: Female[107,108], male[109]; No[21]	Yes: Female[107], male[110]; No[31,37]
Smoking	Yes: Smoker[28,39,111]; No[41]	
Previous surgery	Yes[27]; No[16,112]	
Disease duration	Yes: Longer[49,113,114]	
Phenotype	Yes: Upper digestive tract[27], fistula[27,115], stenosis[115]	
Pharmacokinetic		
Drug concentration	Yes: Low[16,116]	Yes: Low[116]
Antidrug antibodies	Yes[117]	Yes[117]
Weight	Yes: High[16,118,119]	Yes: High[118,119]
Serum albumin	Yes: Low[28,119,120]	Yes: Low[119,120]
Serum γ-globulin	Yes: High[120]	Yes: High[120]
MMP3	Yes: High[123]	Yes: High[123]
FCGR3A	Yes: FCGR3A VV[125]	Yes: FCGR3A VV[125]
HLA	Yes: HLADQA1*05[127]	Yes: HLADQA1*05[127]
Biologic markers		
CRP	Yes: Low[128], high[129]	No[82]
ANA	Yes: ANA+ [130]	Yes: ANA[130]
Fecal calprotectin	Yes: High[28]; No[131]	
Fecal lactoferrin	Yes: High[132]	Yes: High[132]
Genetic markers		
TNFRSF	Yes: TNFRSF1B (rs976881)[86]	
Cytokines		
Interleukin	Yes: OSM (high)[100,133]	Yes: OSM (high)[100]

MMP3: Matrix metalloproteinase 3; FCGR3A: Fcγ receptor type IIIA; HLA: Human leukocyte antigen; CRP: C-reactive protein; ANA: Antinuclear antibody; TNFRSF: Tumor necrosis factor receptor superfamily; OSM: Oncostatin M.

suggest that at least 6 mo of combination therapy is required. Mahmoud *et al*[150] compared different durations of combination therapy in relation to LOR and found no significant difference between durations of combination therapy (< 0.5 years, 0.5-1 year, 1-2 years, and > 2 years); however, durations of combination therapy longer than 2 years were associated with a lower risk of ADAs formation.

Switch within class: In the case of subtherapeutic concentrations with high titers of ADAs, switching within class to another anti-TNF agent should be considered. A retrospective study of IBD showed that when ADA titers of infliximab and adalimumab were > 9 μg/mL and 4 μg/mL, respectively, switching within class achieved a longer duration of response compared with dose intensification[151]. In another study of IBD, switching patients positive for ADAs to another anti-TNF agent achieved a response rate of 92%, whereas dose optimization achieved a response rate of 17%[152]. In cases where the first anti-TNF drug failed, switching to another drug achieved remission in approximately 50% of patients, an effect that has been reported in several other studies[153,154]. Moreover, a systematic review reported that switching to a second anti-TNF agent led to successful induction of remission in 46% of patients with IBD who had failed the first anti-TNF agent[155]. Of note, the previous generation of anti-TNF antibodies increases the risk of the generation of a second anti-TNF antibody in IBD[156]. Therefore, when switching to another anti-TNF agent, a combination of immunosuppressive agents is appropriate[136,157].

Switch out of class: If TL is sufficient with high ADAs, it is recommended that the patient switches to a drug that exerts its effects through another mechanism of action, considering that TNF-α is not the primary pathogenesis. Alternatively, for low TLs with high titers of ADAs, switching out of class is also effective. Subgroup analyses of trials investigating vedolizumab[158], ustekinumab[159,160], and tofacitinib[161] all showed that patients who had failed anti-TNF therapy

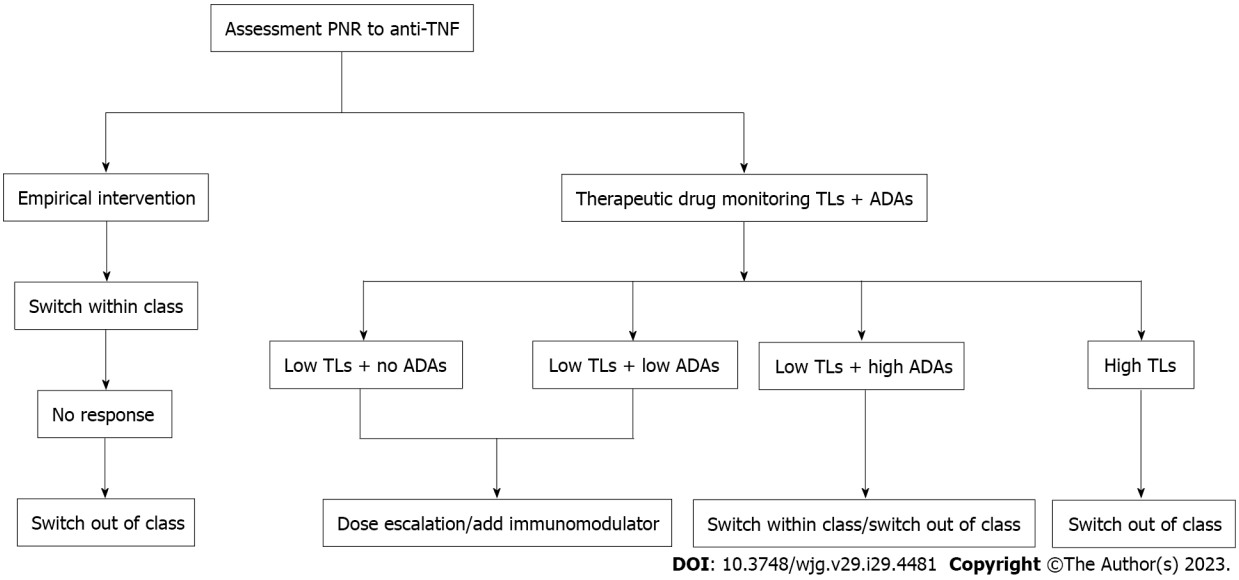

Figure 1 Flow diagram for management of primary nonresponse to tumor necrosis factor antagonists. PNR: Primary nonresponse; TNF: Tumor necrosis factor; TL: Trough level; ADA: Antidrug antibody.

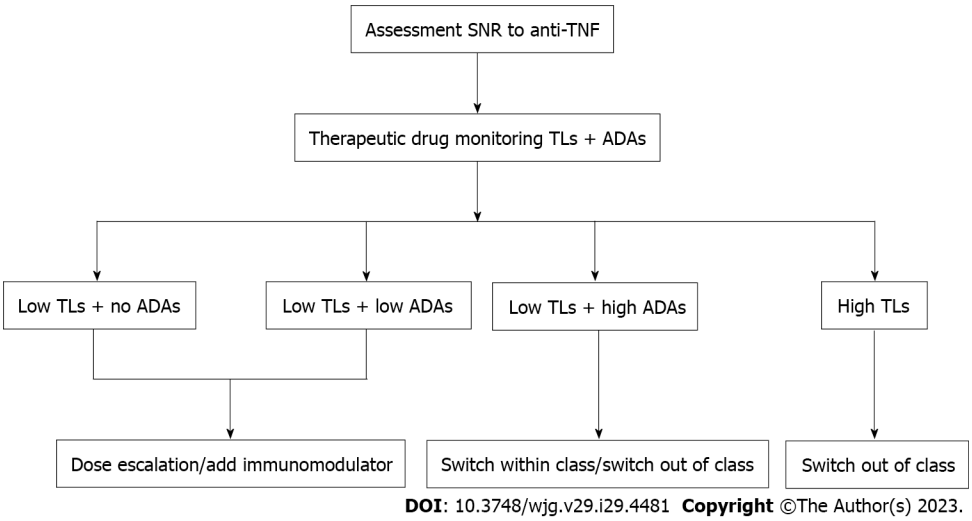

Figure 2 Flow diagram for management of secondary nonresponse to tumor necrosis factor antagonists. SNR: Secondary nonresponse; TNF: Tumor necrosis factor; TL: Trough level; ADA: Antidrug antibody.

benefited from treatment with a novel agent. One study involving 128 CD patients who had failed previous anti-TNF therapy reported that the corticosteroid-free clinical response rates of vedolizumab and ustekinumab treatment at weeks 12, 24, and 52 were 22.7%, 29.7%, 26.8% and 27.1%, 42.4%, 45.9% respectively[162]. Furthermore, propensity score matching concluded that patients who failed anti-TNF therapy benefited more from ustekinumab than vedolizumab[162].

CONCLUSION

IBD is incurable, and anti-TNF therapy plays an important role in IBD. Although existing studies have found that previous surgical history, disease manifestations, drug concentrations, ADAs, serum albumin, ANCA, p-ANCA, ANA, *etc.* have potential predictive effects, to date, there are no practically available indicators that can predict response to TNF antagonists in patients with IBD. Further research is needed to verify the accuracy of existing predictors or discover new biomarkers to achieve personalized treatment for patients with IBD.

TDM forms the core of an optimal strategy for treating IBD. It is recommended to optimize the dose or add immunomodulators when patients with low TLs and no or low titer ADAs. For nonresponders with low TLs and high titer ADAs, switching to another TNF antagonist or biologic agent with a different mechanism can be suggested. When TLs are sufficient, patients can consider switching to another biological agent. In the future, more large randomized

controlled trials are needed to investigate the efficacy of different next-step therapies for IBD patients who do not respond to anti-TNF.

FOOTNOTES

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REVIEW

Bioenergetic alteration in gastrointestinal cancers: The good, the bad and the ugly

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Abstract

Cancer cells exhibit metabolic reprogramming and bioenergetic alteration, utilizing glucose fermentation for energy production, known as the Warburg effect. However, there are a lack of comprehensive reviews summarizing the metabolic reprogramming, bioenergetic alteration, and their oncogenetic links in gastrointestinal (GI) cancers. Furthermore, the efficacy and treatment potential of emerging anticancer drugs targeting these alterations in GI cancers require further evaluation. This review highlights the interplay between aerobic glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (OXPHOS) in cancer cells, as well as hypotheses on the molecular mechanisms that trigger this alteration. The role of hypoxia-inducible transcription factors, tumor suppressors, and the oncogenetic link between hypoxia-related enzymes, bioenergetic changes, and GI cancer are also discussed. This review emphasizes the potential of targeting bioenergetic regulators for anti-cancer therapy, particularly for GI cancers. Emphasizing the potential of targeting bioenergetic regulators for GI cancer therapy, the review categorizes these regulators into aerobic glycolysis/ lactate biosynthesis/transportation and TCA cycle/coupled OXPHOS. We also detail various anti-cancer drugs and strategies that have produced pre-clinical and/or clinical evidence in treating GI cancers, as well as the challenges posed by these drugs. Here we highlight that understanding dysregulated cancer cell bioenergetics is critical for effective treatments, although the diverse metabolic patterns

present challenges for targeted therapies. Further research is needed to comprehend the specific mechanisms of inhibiting bioenergetic enzymes, address side effects, and leverage high-throughput multi-omics and spatial omics to gain insights into cancer cell heterogeneity for targeted bioenergetic therapies.

Key Words: Energy metabolism; Mitochondria; Hypoxia; Oxidative phosphorylation; Glycolysis; Gastrointestinal neoplasms

Core Tip: This review discusses the bioenergetic alteration and metabolic reprogramming in gastrointestinal (GI) cancers, including the interplay between aerobic glycolysis, tricarboxylic acid cycle, and oxidative phosphorylation. The review also highlights potential strategies for targeting bioenergetic regulators for anti-cancer therapy in GI cancers, summarizing the efficacy and challenges of several drugs.

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INTRODUCTION

Cells require energy to carry out their functions, and the most common form of cellular energy is adenosine triphosphate (ATP). This energy is typically produced by oxidative phosphorylation (OXPHOS) in the mitochondria of normal cells[1]. However, in cancer cells, there is a shift in the way energy is generated. Instead of using OXPHOS, cancer cells use glycolysis, a process that results in increased uptake of glucose and secretion of lactate[2]. This phenomenon is known as the Warburg effect and is observed in many types of cancer[3,4]. By understanding the altered energy metabolism in cancer cells, researchers can gain new insights into cancer cell biology and identify potential targets for cancer therapy.

Glycolysis is the process by which glucose is broken down to produce ATP, and it does not require oxygen (Figure 1). Glucose enters cells through glucose transporters and is converted to glucose-6-phosphate (G6P) by hexokinase (HK). Glucose-6-phosphate isomerase (G6PI) converts G6P to fructose-6-phosphate (F6P), which is used in both the glycolytic pathway to generate pyruvate or lactate and the pentose phosphate pathway (PPP) to produce nucleotides and nicotinamide adenine dinucleotide phosphate (NADPH). Phosphofructokinase-1 (PFK1) converts F6P and fructose-2,6-bisphosphate (F2,6BP), a metabolite from a branch driven by fructose-2,6-biphosphatase 3 (PFKBP3), to fructose-1,6-bisphosphate (F1,6BP), which is further processed by aldolase to generate glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). G3P is converted by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to 1,3-bisphosphoglycerate (1,3BPG), which is further converted to 3-phosphoglycerate (3PG) by phosphoglycerate kinase (PGK1). The 3PG is subsequently converted by phosphoglycerate mutase (PGAM) to 2-phosphoglycerate (2PG). The 2PG then serves as a substrate for enolase (ENO) to convert to phosphoenolpyruvate (PEP). Pyruvate kinase isozyme M1/M2 (PKM1/2) catalyzes the conversion of PEP to pyruvate, which can be converted to acetyl-CoA or lactate. This process generates NAD+ from NADH, which is important for the continuation of the glycolysis process. Although glycolysis itself does not require oxygen, the fate of the pyruvate produced by glycolysis depends on the availability of oxygen, and the overall efficiency of ATP production is much higher when oxygen is present[5].

Pyruvate, a product of glycolysis, enters the mitochondria where it is converted to acetyl-CoA. The resulting acetyl-CoA can then enter the tricarboxylic acid (TCA) cycle, also known as the Krebs cycle, which plays a pivotal role in generating ATP through the electron transport chain (ETC). The TCA cycle completes the breakdown of glucose by breaking down acetyl-CoA into carbon dioxide (CO_2) and water, releasing energy in the form of NADH and flavin adenine dinucleotide (FADH2). NADH and FADH2 donate their electrons to the ETC at Complex I and II, respectively. The ETC, specifically Complexes I-IV, transfers electrons from NADH and FADH2 to generate a proton gradient across the inner mitochondrial membrane. This gradient is then used by ATP synthase to produce ATP. Complex I, also known as NADH dehydrogenase or NADH ubiquinone oxidoreductase, is the largest of the five mitochondrial complexes and marks the initiation of the ETC[6]. Electrons are transferred from Complex I to coenzyme Q (CoQ) across the inner mitochondrial membrane and then from CoQ to Complex III, although an alternative pathway exists *via* Complex II, succinate dehydrogenase (SDH)[7,8]. Following reduction of succinate by Complex II, electrons are transported to CoQ and then transferred to Complex III. Complex III and cytochrome c transfer electrons to Complex IV, cytochrome c oxidase (COX). The ETC complexes act as proton pumps, creating an electrochemical gradient across the inner mitochondrial membrane, and this energy is harnessed by Complex V, ATP synthase, which generates ATP by using the energy from the movement of protons down their electrochemical gradient. This whole process is known as OXPHOS and is a time-consuming process compared to glycolysis, but is the most efficient way to generate ATP in the cell, producing up to 36-38 ATP molecules per glucose molecule. Complexes I-IV are known as the ETC, while Complex V (ATP synthase) does not (Figure 1). Except for Complex II, all OXPHOS-related complexes are partially encoded by mitochondrial DNA (mtDNA)[9]. Unfortunately, OXPHOS also produces reactive oxygen species (ROS) as a byproduct,

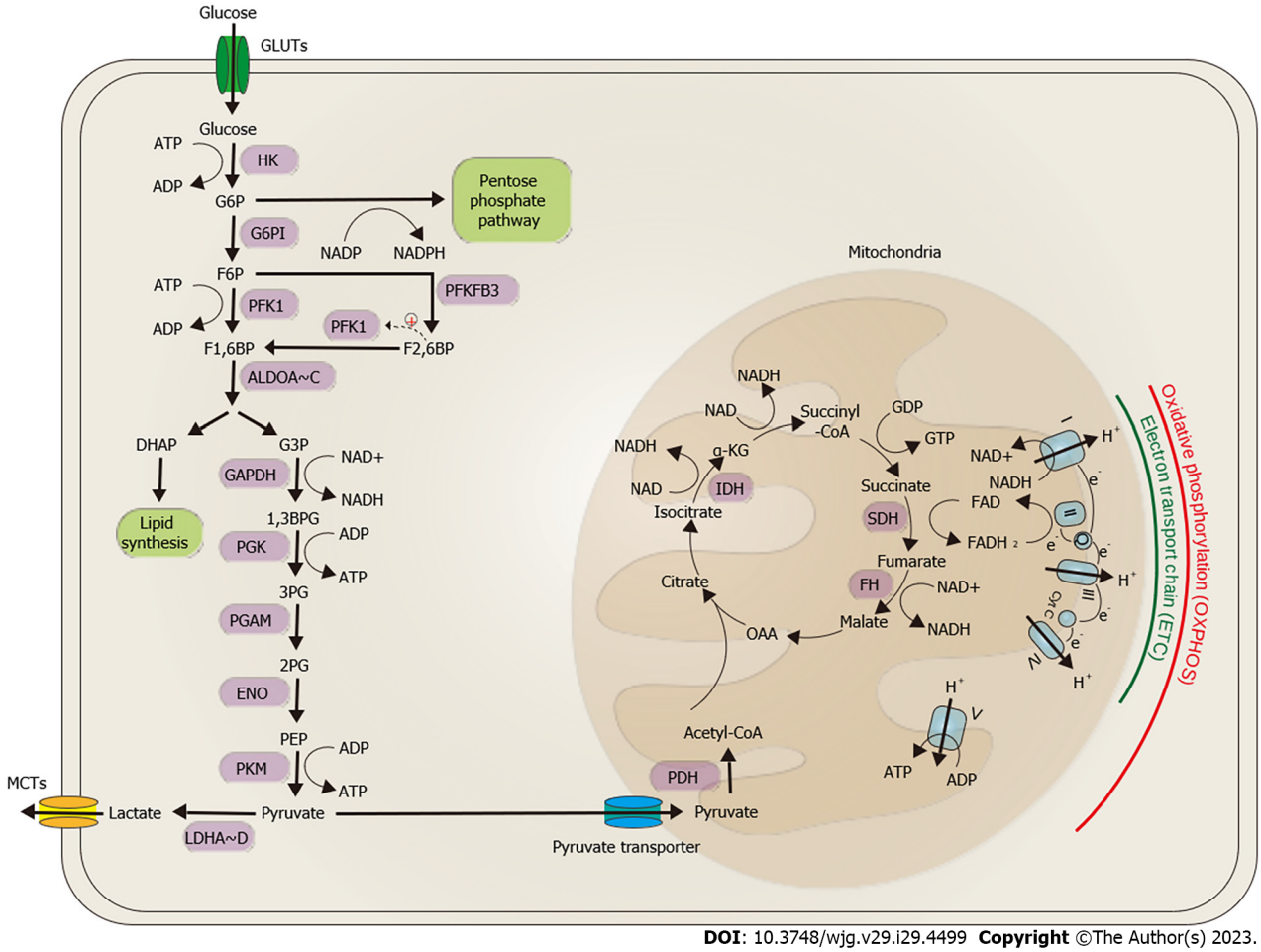

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Figure 1 Illustration of the pathway of glucose metabolism. Glucose is taken up by cells and undergoes a series of reactions to convert it to pyruvate *via* the process of glycolysis. Pyruvate can then enter the tricarboxylic acid cycle in the mitochondria to produce energy, or it can be converted to lactate in the cytosol under anaerobic conditions. The key enzymes involved in these reactions are highlighted in pale-purple, and linked pathways are depicted in pale-green. The mitochondrial complexes that are critical for oxidative phosphorylation and adenosine triphosphate production are shown in pale-blue. GLUT: Glucose transporter; HK: Hexokinase; G6P: Glucose-6-phosphate; G6PI: Glucose-6-phosphate isomerase; F6P: Fructose-6-phosphate; NADPH: Nicotinamide adenine dinucleotide phosphate; PFK1: Phosphofructokinase-1; F2,6BP: Fructose-2,6-bisphosphate; PFKBP3: Fructose-2,6-biphosphatase 3; F1,6BP: Fructose-1,6-bisphosphate; G3P: Glyceraldehyde-3-phosphate; DHAP: Dihydroxyacetone phosphate; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; 1,3BPG: 1,3-bisphosphoglycerate; 3PG: 3-phosphoglycerate; PGK: Phosphoglycerate kinase; PGAM: Phosphoglycerate mutase; 2PG: 2-phosphoglycerate; ENO: Enolase; PEP: Phosphoenolpyruvate; PKM1/2: Pyruvate kinase isozyme M1/M2; LDH: Lactate dehydrogenase; MCT: Monocarboxylate transporter family; PDH: Pyruvate dehydrogenase; IDH: Isocitrate dehydrogenase; α-KG: α-ketoglutarate; OAA: Oxaloacetate; SDH: Succinate dehydrogenase; FH: Fumarate hydratase; I: Mitochondrial complex I; II: Mitochondrial complex II; III: Mitochondrial complex III; IV: Mitochondrial complex IV; V: Mitochondrial complex V; Q: Co-enzyme Q; cyto C: Cytochrome *c*; ATP: Adenosine triphosphate; ADP: Adenosine diphosphate; FADH2: Flavin adenine dinucleotide; e⁻: Electrons.

which can cause damage to mitochondrial or nuclear DNA and activate oncogenic signaling pathways, potentially leading to diseases and carcinogenesis[10-12]. Mutations in mtDNA are also implicated in cancer[13]. Overall, the process of OXPHOS is vital for cellular energy production, but careful regulation is necessary to prevent the damaging effects of ROS production.

In cancer cells, certain enzymes and molecules involved in the conversion of glucose to energy are upregulated, which provides an attractive target for anti-cancer therapies[14]. Disrupting this process could prevent cancer cells from producing energy and lead to their death. In addition to the upregulation of these enzymes, alterations in certain mitochondrial enzymes and oncometabolites have been identified in cancer cells. Oncometabolites are small molecules that are produced in cancer cells and contribute to their growth and proliferation[15]. These alterations can be caused by genetic and epigenetic changes in the genes involved in energy production[13,16]. Recent research has focused on understanding these bioenergetic alterations in gastrointestinal (GI) cancers, such as esophageal cancer (ESCA), gastric cancer (GC), hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), pancreatic cancer (PAC), and colorectal cancer (CRC). Understanding these specific metabolic changes in cancer cells can provide insight into developing more effective targeted therapies for GI cancers. In addition to the potential for targeted therapy, these metabolic changes could also serve as biomarkers for cancer diagnosis and prognosis. By identifying alterations in the genes and molecules involved in energy production, clinicians may be able to more accurately diagnose and predict the course of the disease. Overall, understanding the bioenergetic alterations in cancer cells is a promising avenue for developing new therapies and improving cancer diagnosis and treatment. In this review, we summarize the latest findings on bioenergetic alterations in

various GI cancers, and discuss the potential therapeutic strategies that target these alterations. Such strategies may include inhibitors of specific enzymes or molecules involved in energy production, as well as interventions aimed at modulating the metabolic environment of cancer cells. Further research in this area could lead to new and more effective treatments for GI cancers.

BIOENERGETIC ALTERATION AND THE WARBURG EFFECT

The process of bioenergetic alteration in cancer involves changes in the way cancer cells generate energy. One well-known component of bioenergetic alteration is the Warburg effect. This phenomenon describes how cancer cells prefer to use glucose fermentation to produce energy even in the presence of oxygen[2]. This process, called aerobic glycolysis, is less efficient than mitochondrial OXPHOS in terms of ATP production[17,18]. However, it has been noted that respiration alone can maintain tumor viability, suggesting that glucose and oxygen must be eliminated to kill cancer cells by depriving them of energy[2]. The underlying mechanisms of the Warburg effect have been investigated for decades. Otto Warburg originally proposed that mitochondrial dysfunction could be responsible for aerobic glycolysis[19]. This theory was later confirmed and explored by another group that demonstrated the Warburg effect could be caused by an imbalance of intracellular pH and mitochondrial ATPase dysfunction[20]. Moreover, it was observed that aerobic glycolysis could be controlled by cascade signaling mediated by growth factors and oncogenes, questioning whether the Warburg effect was a mere bystander in the pathogenesis of cancer[21-24]. It was not until later that the Warburg effect was discovered to be crucial for tumor growth in genetic and pharmacological studies[25,26].

Scientists have been trying to understand why cancer cells prefer aerobic glycolysis to mitochondrial OXPHOS for decades, given that the ATP generated by aerobic glycolysis is much lower than that produced by mitochondrial OXPHOS[27-29]. Recent studies have shed light on this phenomenon. For example, when changes in the cellular environment increase ATP demand through alteration of ATP-dependent membrane activity, aerobic glycolysis increases rapidly and OXPHOS remains unchanged[30]. Another study showed high aerobic glycolysis as a metabolic strategy which cancer cells use to optimally respond to fluctuating energy availability[31]. Together, this literature suggests that the Warburg effect is a metabolic strategy that allows flexibility among cancer cells under an unpredictable tumor microenvironment.

THE DYNAMIC INTERPLAY BETWEEN OXPHOS AND AEROBIC GLYCOLYSIS

Not all pyruvate produced during glycolysis is converted to lactate. Indeed, a significant amount of pyruvate can enter the TCA cycle for oxidation and further metabolism. The intermediates generated during the TCA cycle, such as NAD+/NADH and NADP+/NADPH, can continue to enter the OXPHOS pathway, which can further generate bioenergy[32,33]. Although the role of the Warburg effect in cancers remains controversial, interfering with tumor metabolism and targeting both aerobic glycolysis and mitochondrial OXPHOS pathways have been shown to be necessary[34-37]. It is evident from current literature that there exists crosstalk between aerobic glycolysis, the TCA cycle, and coupled OXPHOS, suggesting cooperative and competitive roles in cancer. Interestingly, some studies suggest that targeting mitochondrial metabolism alone may not be sufficient to inhibit tumor growth, as cancer cells can redirect their metabolism to rely on other energy sources. In such cases, blocking both the glycolytic and mitochondrial pathways may be necessary to prevent cancer cell growth[34-37]. Therefore, a better understanding of the metabolic pathways in cancer cells and their interactions is required to develop effective cancer therapies.

Although the exact molecular mechanism that triggers the Warburg effect in cancer remains unclear, multiple hypotheses have been proposed, including the involvement of tumor suppressors (*e.g.*, p53) and oncogenes (*e.g.*, PI3K, AKT, mTOR), all of which appear to converge on the role of hypoxia-inducible transcription factors (HIFs), particularly HIF-1. HIF-1 is a transcription factor that regulates cellular responses to oxygen deprivation, and it was initially identified as a protein that is present only under hypoxic conditions[38-41]. However, it was later discovered that HIF-1 can also be stabilized under normoxia in a microenvironment with high lactate concentration[42,43]. Under normal conditions, HIF-1 α, a subunit of HIF-1, is targeted for degradation by prolyl hydroxylases (PHDs), which utilize molecular oxygen to hydroxylate HIF-1α, leading to its recognition by the von Hippel-Lindau tumor suppressor (VHL), and degradation *via* proteasome-mediated pathways[44-47].

HIF-1 regulates the expression of several key glycolytic enzymes, such as glucose transporter-1 (GLUT1), GLUT3, HK, aldolase A (ALDOA), PGK1, PKM1/2, ENO1, pyruvate dehydrogenase kinase (PDKs), and lactate dehydrogenase subunit A (LDHA), by directly promoting their expression[48-54]. This leads to an increased level of pyruvate, the final product of glycolysis. However, it is important to note that cancer cells with high glycolytic activity are not guaranteed to catabolize all pyruvate to lactate, as significant amounts of pyruvate can enter the TCA cycle for oxidation and metabolism. In cancer cells, it is suggested that the HIF-1 induced increased expression of PDKs can inhibit the function of pyruvate dehydrogenase (PDH), which blocks pyruvate entry into the TCA cycle and promotes lactate production. Since HIF-1 also promotes the expression of LDHA, an important subunit of LDH necessary for lactate biosynthesis from pyruvate, it is thought to be crucial in cancers affecting terminal lactate levels[55] (Figure 2). Therefore, HIF-1 plays a significant role in the Warburg effect, which may have implications for cancer diagnosis and treatment. While the precise molecular mechanism behind the Warburg effect remains to be elucidated, the involvement of HIF-1 is clear. Understanding the interplay between HIF-1, glycolysis, and OXPHOS in cancer cells may lead to the development of novel cancer therapies that target both pathways.

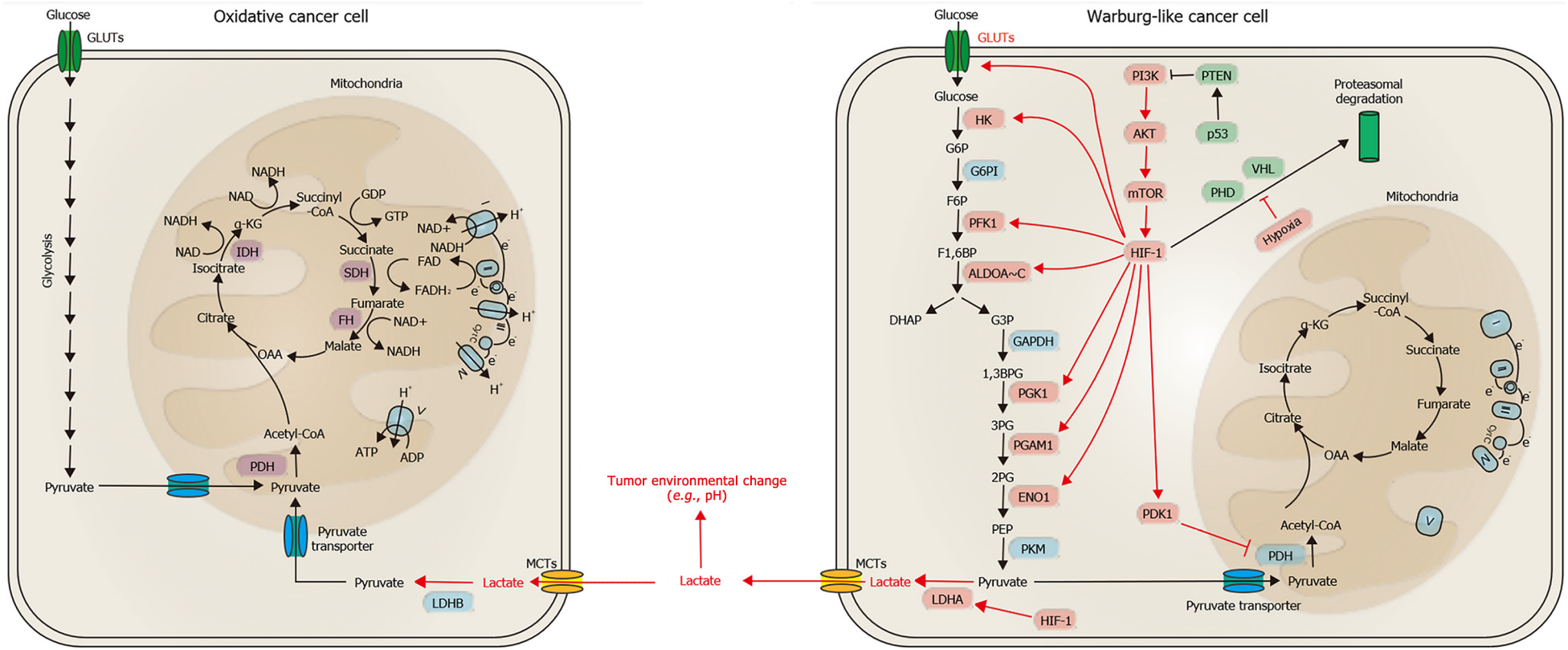

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Figure 2 The complex interplay between glycolysis and oxidative phosphorylation in cancer cells. This figure highlights the signaling networks and metabolic regulation in both Warburg-like and oxidative cancer cells. p53 induces PTEN and represses PI3K activity, which inhibits glycolysis and opposes the Warburg effect. Hypoxia and the subsequent activation of hypoxia-inducible factor 1 (HIF-1) play a crucial role in modulating various aspects of cancer cell metabolism, including glycolysis, lactate production, and the tricarboxylic acid (TCA) cycle. Hypoxia counteracts the degradation of HIF-1 by prolyl hydroxylases and von Hippel-Lindau, which stabilizes and activates HIF-1. HIF-1 then transcriptionally activates genes such as hexokinase, phosphofructokinase-1, aldolase A, PGK1, PGAM1, ENO1, and LDHA, as indicated by the red arrows. During glycolysis, excessive lactate can be exported to the extracellular environment, leading to microenvironmental changes such as a lower pH. Intracellular lactate can also be transferred to adjacent cells and re-converted to pyruvate, which can enter the TCA cycle and drive oxidative phosphorylation in oxidative cancer cells. GLUT: Glucose transporter; HK: Hexokinase; G6P: Glucose-6-phosphate; G6PI: Glucose-6-phosphate isomerase; F6P: Fructose-6-phosphate; NADPH: Nicotinamide adenine dinucleotide phosphate; PFK1: Phosphofructokinase-1; F2,6BP: Fructose-2,6-bisphosphate; PFKBP3: Fructose-2,6-biphosphatase 3; F1,6BP: Fructose-1,6-bisphosphate; G3P: Glyceraldehyde-3-phosphate; DHAP: Dihydroxyacetone phosphate; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; 1,3BPG: 1,3-bisphosphoglycerate; 3PG: 3-phosphoglycerate; PGK: Phosphoglycerate kinase; PGAM: Phosphoglycerate mutase; 2PG: 2-phosphoglycerate; ENO: Enolase; PEP: Phosphoenolpyruvate; PKM1/2: Pyruvate kinase isozyme M1/M2; LDH: Lactate dehydrogenase; MCT: Monocarboxylate transporter family; PDH: Pyruvate dehydrogenase; IDH: Isocitrate dehydrogenase; α-KG: α-ketoglutarate; OAA: Oxaloacetate; SDH: Succinate dehydrogenase; FH: Fumarate hydratase; I: Mitochondrial complex I; II: Mitochondrial complex II; III: Mitochondrial complex III; IV: Mitochondrial complex IV; V: Mitochondrial complex V; Q: Co-enzyme Q; cyto C: Cytochrome *c*; HIF-1: Hypoxia-inducible factor 1; PHD: Prolyl hydroxylases; VHL: Von Hippel-Lindau.

The concept of lactate as a metabolic waste product has been revised with the latest findings in lactate metabolism and transport. It is now known that lactate can serve as an alternative fuel for certain types of cells, including cancer cells[56,57]. In cancer, the excess lactate is transported between the intracellular and extracellular matrix by the monocarboxylate transporter family (MCT1-4), which depends on the gradients of the protons and monocarboxylate ions[58,59]. Imported extracellular lactate can be converted to pyruvate *via* LDH primarily composed by the LDHB subunit[60,61]. In oxidative cancer cells with a functional TCA cycle and OXPHOS, pyruvate can be further converted to acetyl-CoA through PDH, thus linking aerobic glycolysis and OXPHOS[62,63]. It has been demonstrated that HIF-1 and downstream oncometabolite lactate play causal roles in these regulatory events. Therefore, current findings provide a possible explanation for the Warburg effect and crosstalk of bioenergetic homeostatic transition between aerobic glycolysis and OXPHOS observed in cancer. The importance of lactate in cancer metabolism and its potential as a therapeutic target have been recognized by others in the field. Thus, a better understanding of the metabolic pathways and their interactions could lead to the development of new strategies for cancer treatment.

THE LINK BETWEEN HYPOXIA-RELATED ENZYMES, BIOENERGETIC CHANGES, AND GI CANCER: A GENETIC AND EPIGENETIC PERSPECTIVE

Cancer cells often undergo a metabolic shift characterized by increased glycolysis and decreased mitochondrial respiration, a phenomenon known as the Warburg effect. This metabolic reprogramming has been linked to the activity of HIF-1 under low-oxygen conditions[64,65]. Genetic and epigenetic alterations in HIF-1 regulatory genes contribute to the development of the Warburg effect in cancer. Methylation-induced epigenetic changes can drive transcriptional changes, leading to impaired expression of key enzymes involved in bioenergetic homeostasis. Additionally, mutations in nuclear and mitochondrial genomes may cause a loss of function or decreased expression of glycolytic/OXPHOS enzymes. Therefore, mutations, transcriptional changes, or epigenetic alterations that enhance HIF-1 stability or activity can lead to increased aerobic glycolysis, resembling the Warburg effect (Table 1).

Studies have found that alterations in PHD enzymes, which target HIF-1 for degradation, contribute to cancer development and progression. Reduced expression or loss-of-function due to PHD2 mutations lead to constitutive activation of HIF-1 and have been found to stimulate HCC and CC development and progression in mouse models[66,67]. In contrast, decreased PHD1-3 expression correlates with increased HIF-1 and vascular endothelial growth factor (VEGF) levels, invasive tumor behavior, and poor prognosis in certain GI cancers such as HCC[68], GC[69-71], and CRC[72]. Interestingly, the opposite effect has been observed in patients with PAC[73]. Another protein involved in HIF-1 stabilization, VHL, also plays a role in GI cancers. Mutations or promoter methylation within the *VHL* gene lead to increased cytoplasmic HIF-1 levels and an unfavorable prognosis in patients with PAC and CRC[74,75]. However, the general status of VHL protein expression in GI cancers remains unclear, with the exception of HCC, whose levels have been shown to decrease, and low levels correlate with poor prognosis[76]. Further investigation is needed to determine the impact of mutations, genetic, or epigenetic alterations in these hypoxia-associated enzymes on bioenergetic alterations in GI cancers, since understanding the mechanisms behind the Warburg effect and the role of HIF-1 regulatory genes could potentially provide new therapeutic targets for treating GI cancers.

THE LINK BETWEEN MITOCHONDRIAL AND NUCLEAR GENE EXPRESSION, BIOENERGETIC HOMEOSTASIS, AND THE PROGRESSION OF GI CANCERS

Cancer development and progression are often accompanied by changes in cellular metabolism that contribute to tumor growth and survival. In addition to genetic and epigenetic alterations in hypoxia-associated regulatory enzymes that promote aerobic glycolysis, emerging evidence suggests that changes in nuclear-encoded genes for enzymes and subunits involved in OXPHOS and the TCA cycle may also play a role in driving the switch to glycolysis and altering bioenergetic homeostasis in cancer. Studies have shown that changes in the expression of key enzymes involved in OXPHOS, such as cytochrome *c* oxidase (COX) and ATP synthase, as well as the TCA cycle enzymes isocitrate dehydrogenase (IDH), fumarate hydratase (FH), and succinate dehydrogenase (SDH), may contribute to glycolysis transition and cancer progression[77-80]. Furthermore, mutations and copy number alterations in mtDNA have also been identified as important factors in the development and progression of GI cancer by altering bioenergetic homeostasis[81]. These emerging factors and their potential contribution to the complex mechanisms underlying the progression of GI cancer are discussed in more detail in the following sections.

ROLE OF MITOCHONDRIAL-NUCLEAR ENCODED COX SUBUNITS IN BIOENERGETIC CHANGES AND PROGRESSION OF GI CANCERS

The COX complex, also known as respiratory chain complex IV, is a multi-subunit enzyme complex, consisting of 14 subunits, and a vital component of the final step in the mitochondrial ETC responsible for catalyzing the transfer of electrons from cytochrome c to oxygen, a crucial step in the process of OXPHOS[82]. Recent studies have shown that alterations in the expression of both mtDNA-encoded and nuclear-encoded COX subunits are associated with tumori-

Table 1 Genetic and epigenetic alterations in hypoxia-related enzymes correlated with the development and progression of gastrointestinal cancers

Cancer type	Gene	Type of change	Consequence	Model	Ref.
HCC and CCA	PHD2	Haplo-deficiency	Stabilized HIF-1 and promoted carcinogenesis and progression of HCC/CCA	Mice	[66, 67]
HCC	PHD3	Reduced tumor level	Correlated with elevated levels of HIF-1, aggressive tumor behavior, and a poor prognosis in HCC patients	HCC patient	[68]
GC	PHD3	Reduced tumor level	Correlated negatively with tumor size and stage, as well as HIF-1 and VEGF expression	GC patient	[69, 70]
GC	PHD2	Reduced tumor level	Correlated with shortened overall survival	GC patient	[71]
CRC	PHD1-3	Reduced tumor level	Although not correlated with HIF-1 expression, PHD2 was the only factor found to be associated with unfavorable overall survival	CRC patient	[72]
PAC	PHD1-3	Increased tumor level	PHD1-3 expression was elevated, and specifically PHD3 expression was found to be associated with unfavorable overall disease-specific survival	PAC patient	[73]
PAC	VHL	Promoter methylation or deletion of VHL	Correlated with decreased VHL expression and poor prognosis	PAC patient	[74]
CRC	VHL	*VHL* mutation	Elevated cytoplasmic expression of HIF-1 in tumors	CRC patient	[75]
HCC	VHL	Reduced tumor level	Negative VHL expression was correlated with an unfavorable prognosis	HCC patient	[76]

ESCA: Esophageal cancer; GC: Gastric cancer; HCC: Hepatocellular carcinoma; CCA: Cholangiocarcinoma; PAC: Pancreatic cancer; CRC: Colorectal cancer; PHD: Prolyl hydroxylase; VHL: Von Hippel-Lindau tumor suppressor.

genesis, cancer progression, and bioenergetic homeostasis in cancer. In GI cancers, alterations in the expression of the mitochondrial-nuclear encoded subunits of the COX complex have been implicated in driving disease progression. Studies have shown that the overall levels of the COX complex are increased in GI cancers, and higher levels have been associated with poor clinical outcomes[83,84]. Of the three mtDNA-encoded core subunits essential for the basic functions of the COX complex, including MTCO1, MTCO2, and MTCO3[85], MTCO1 is the most frequently investigated in GI cancers (Table 2). In ESCA, MTCO1 expression was found to be elevated but did not correlate with clinicopathological variables or survival[86]. On the other hand, elevated levels of MTCO1 were associated with diffuse GC types, suggesting a link between MTCO1 expression and GC carcinogenesis, de-differentiation, and distant metastasis[87,88]. In contrast, defective MTCO1 expression was observed in patients with HCC and CCA, while MTCO1 levels have been shown to predict postoperative survival in patients with HCC[89,90]. Elevated MTCO3 levels have been observed only in HCC, especially among patients with hepatitis B virus (HBV)-related HCC. This is likely due to the ability of the HBV X protein (HBx) to interact and increase MTCO3 expression[91,92]. Additionally, genetic variants identified within MTCO1 and MTCO3 are associated with increased carcinogenic risk in CRC[93,94], GC[95], and HCC[96], possibly due to reduced COX activity leading to intrinsic proton leak and a reduction in overall bioenergetic production efficiency[93,94]. However, studies on the expression or genetic variation of MTCO2 in GI cancers are relatively few and need further investigation.

While the three core mtDNA-encoded COX subunits have been extensively studied, 11 nuclear-encoded protein subunits are also required for the full functionality of the COX complex[97]. Of these 11 subunits, six can be replaced by isoforms, leading to heterogeneity in the composition and activity of this large complex[98]. In GI cancers, altered expression of nuclear-encoded COX subunits has been shown to play a crucial role in the switch to glycolysis and the promotion of tumor growth and progression (Table 2). For example, in ESCA, the silencing of COX4I1 and COX5B has been shown to promote bioenergetic changes and increased aggressiveness of ESCA cells *in vitro*[99]. In HCC and CRC, COX5B levels were found to correlate with prognosis, and changes in COX5B expression were associated with alterations in bioenergetics, cell proliferation, tumor growth, migration, and chemosensitivity. HCC and CRC, however, showed different COX5B expression patterns[100-102]. Similarly in CRC, increased COX4I2 has been shown to promote cell proliferation, migration, tumorigenesis, and angiogenesis[103]. COX6C and COX6B2 were also found to be increased in PAC, with changes in expression levels of COX6C affecting COX activity and cell growth *in vitro*. Meanwhile, COX6B2 levels were associated with prognosis, metastatic potential in PAC cells, and altered bioenergetic homeostasis[104,105].

The roles of remaining subunits in GI cancer are currently unknown, and studies focusing on the level of nuclear-encoded COX subunit in GI cancer largely suggest that altered expression leads to decreased OXPHOS activity in a Warburg effect-like phenotype. Increased GI cancer growth and/or progression is also suggested. Together, these findings highlight the crucial role COX subunits play in GI cancer progression and underscore the need for continued research. The identification of altered COX subunit expression and function may lead to the development of novel therapeutic targets for the treatment of GI cancers. Therefore, further research on the COX complex and its subunits is

Table 2 Defects in cytochrome c oxidase subunits correlated with bioenergetic alterations and the growth or progression of gastrointestinal cancers

Type	Gene	Type of defect	Consequence	Model	Ref.
GC	Full COX complex	Increased expression	Correlated with poor prognosis	GC patient	[83]
CRC	Full COX complex	Increased expression	May be involved in the initiation of carcinogenesis, but not in cancer progression	CRC patient	[84]
ESCA	MTCO1	Increased expression	There is no correlation with clinical variables or survival	ESCA patient	[86]
GC	MTCO1	Increased expression	Correlated with gastric tumorigenesis, dedifferentiation, and distant metastasis, but showed no significant correlation with prognosis	GC patient	[87,88]
HCC	MTCO1	Reduced expression	Correlated with postoperative prognosis	HCC patient	[89]
CCA	MTCO1	Reduced expression	Reduced MTCO1 correlates with increased VDAC1 expression but not with other clinicopathological factors	CCA patient	[90]
HCC	MTCO3	Increased expression	HBx interacted with MTCO3, leading to an increase in MTCO3 expression levels and an enhancement in OXPHOS activity	Cell line	[91,92]
CRC	MTCO1	Genetic variation	The Gly125Asp substitution in MTCO1 correlated with an increased risk of CRC and caused proton leak in COX	CRC patient	[93,94]
GC	MTCO3	Genetic variation	Polymorphisms at mtDNA positions 9540 and 9548 correlated with an increased risk of GC	GC patient	[95]
HCC	MTCO3	Genetic variation	Polymorphisms at mtDNA position 9545 correlated with an increased risk of HCC	HCC patient	[96]
ESCA	COX4I1	Expression silenced	Promotes alterations in cellular bioenergetics and increases cancer cell aggressiveness	ESCA Cell line	[99]
ESCA	COX5B	Expression silenced	Promotes alterations in cellular bioenergetics and increases cancer cell aggressiveness	ESCA Cell line	[99]
HCC	COX5B	Increased in tumor	Correlated with prognosis, regulated bioenergetic alterations, and influenced cell proliferation, tumor growth, and migration	HCC patient, cell line, mouse model	[100]
CRC	COX5B	Reduced in tumor	Correlated with prognosis, modulated COX activity, and controlled cell proliferation, apoptosis, and response to chemotherapy	CRC patient and cell line	[101,102]
CRC	COX4I2	Increased in tumor	Promoted cell proliferation, migration, tumorigenesis, and angiogenesis	CRC patient and cell line	[103]
PAC	COX6C	Increased expression	Modulated COX activity and cell proliferation	PAC cell line	[104]
PAC	COX6B2	Increased in tumor	Correlated with prognosis, and modulated cancer cell metastatic potential, and altered bioenergetic homeostasis	PCA patient and cell line	[105]

COX: Cytochrome *c* oxidase; GI: Gastrointestinal; ESCA: Esophageal cancer; GC: Gastric cancer; HCC: Hepatocellular carcinoma; CCA: Cholangiocarcinoma; PAC: Pancreatic cancer; CRC: Colorectal cancer; MTCO1: Mitochondrially encoded cytochrome *c* oxidase I; MTCO2: Mitochondrially encoded cytochrome *c* oxidase II; MTCO3: Mitochondrially encoded cytochrome *c* oxidase III; COX4I1: Cytochrome *c* oxidase subunit 4I1; COX4I2: Cytochrome *c* oxidase subunit 4I2; COX5B: Cytochrome *c* oxidase subunit 5B; COX6C: Cytochrome *c* oxidase subunit 6C; COX6B2: Cytochrome *c* oxidase subunit 6B2.

needed to fully elucidate their role in GI cancer.

THE ROLE OF ATP SYNTHASE SUBUNITS IN DRIVING BIOENERGETIC CHANGES AND GI CANCER PROGRESSION

ATP synthase, also known as Complex V, is a crucial mitochondrial protein complex that plays a vital role in cellular ATP synthesis. The F1 beta-catalytic subunit (ATP5F1B) is a critical component that has been extensively studied to find a significant reduction in various cancer types, including GI cancers[106] (Table 3). However, the expression patterns of ATP5F1B in patients with GC remain controversial. While one study reported increased ATP5F1B expression in tumors,

Table 3 Implications of defects in adenosine triphosphate synthase subunits on bioenergetic alterations and the development or progression of gastrointestinal cancer

Type	Gene	Type of defect	Consequence	Model	Ref.
GC	ATP5F1B	Increased in tumor	Higher ATP5B expression correlated with poor prognosis. Over-expression of ATP5F1B increased intracellular and extracellular ATP levels, cell proliferation, migration, and invasion	GC patient, cell line, and xeno-transplantation mouse model	[107]
GC	ATP5F1B	Reduced in tumor	Reduced ATP5F1B expression correlated with elevated glycolytic enzyme levels	GC patient	[108]
HCC	ATP5F1B	Reduced in tumor	Reduced ATP5F1B expression correlated with impaired OXPHOS	HCC patient	[109, 110]
ESCA	ATP5F1B	Reduced in tumor	Reduced ATP5F1B expression correlated with elevated glycolytic enzyme levels	ESCA patient	[108]
CRC	ATP5F1B	Reduced in tumor	Reduced ATP5F1B expression correlated with poor prognosis in CRC patients	CRC patient	[109]
PAC	ATP5F1B	Reduced in tumor	Unknown	PAC patient and cell line	[111]
CRC	ATP5F1A	Increased in liver metastasized tumor	Silencing of ATP5F1A inhibited cell invasion and reduced cell proliferation in CRC cancer cells	CRC patient and cell line	[112]
CRC	ATP5F1E	Increased in tumor	Higher ATP5E levels correlated with poor prognosis. Silencing of ATP5F1E inhibited cancer cell migration and invasion *in vitro*, and distal metastasis *in vivo*	CRC patient, cell line, and tail vein injected mouse model	[113]
CRC	ATP5F1D	Increased in liver metastasized tumor	Higher ATP5F1D expression correlated with poor prognosis, and silencing of ATP5F1D inhibited cell invasion	CRC patient and cell line	[112]

ATP: Adenosine triphosphate; GI: Gastrointestinal; ESCA: Esophageal cancer; GC: Gastric cancer; HCC: Hepatocellular carcinoma; CCA: Cholangiocarcinoma; PAC: Pancreatic cancer; CRC: Colorectal cancer; ATP5F1A: ATP synthase F1 subunit alpha; ATP5F1B: ATP synthase F1 subunit beta; ATP5F1D: ATP synthase F1 subunit delta; ATP5F1E: ATP synthase F1 subunit epsilon.

correlating with poor prognosis[107], consistent findings from other GI cancer studies indicate that decreased ATP5F1B expression results in reduced ATP production efficiency from OXPHOS and a subsequent shift towards the glycolysis-dependent Warburg effect phenotype[108-111]. These findings highlight the critical role of ATP synthase in GI cancer progression, suggesting that mitochondrial defects in ATP synthesis may contribute to the bioenergetic alterations observed in these cancers.

Apart from the F1 beta-subunit, other subunits of the ATP synthase F1 region have been implicated as crucial to CRC carcinogenesis/progression. Interestingly, in contrast to the finding that ATP5F1B generally decreases in tumors, ATP5F1A, ATP5F1E, and ATP5F1D were found to be increased in patients with CRC. Moreover, higher levels correlated with poorer prognosis as well as increased risk of CRC liver metastasis[112,113]. Currently, there are no reports on the expression patterns or role of ATP synthase subunits in CCA. The mechanisms underlying opposing expression patterns in ATP synthase subunits are thus unknown pending further investigation.

To provide more insight into the development of novel therapeutic targets for the treatment of GI cancers, further research on ATP synthase expression and function is necessary. In this regard, potential avenues of research may focus on clarifying the controversial findings regarding ATP5F1B expression patterns in GC and elucidating the mechanisms underlying these opposing expression patterns seen in differing ATP synthase subunits in CRC. Such research may uncover novel therapeutic targets, leading to improved treatment outcomes.

LINKING IDH, FH, AND SDH TO BIOENERGETICS AND GI CANCER PROGRESSION

Fumarate and succinate are critical metabolites that are produced during the TCA cycle, which is an essential process for energy production in cells. While these metabolites are important for normal cellular function, they have been shown to act as oncometabolites in various types of cancer by inducing pseudohypoxia[114]. Specifically, aberrant fumarate and succinate accumulation resulting from mutations or abnormal expression in FH and SDH, respectively, can impede the production of α-ketoglutarate in the TCA cycle, which is a key substrate in tumor suppression pathways. Similarly, mutations in IDH enzymes, which are responsible for α-ketoglutarate synthesis, can directly reduce the levels of α-ketoglutarate. This reduction in α-ketoglutarate can limit the availability of substrate for the hydroxylation of HIF-1 by PHDs for subsequent degradation by the proteasome. Consequently, stabilized HIFs activate the transcription of genes involved in cancer-related processes such as angiogenesis, glucose metabolism, and cell proliferation, thereby promoting cancer development and progression[114].

In addition to their effects on HIFs, high levels of fumarate and succinate have been shown to cause abnormal methylation of DNA and histones, leading to dysregulation of gene expression and cell function. This is due to

attenuation of enzymes responsible for DNA and histone demethylation such as tet-eleven translocation methyl-cytosine dioxygenase (TET) and lysine demethylase (KDM, also known as the Jumonji C domain-containing histone demethylase, JHDM)). Dysregulation of gene expression, increased carcinogenicity, and cancer progression can result from decreased α -ketoglutarate under high fumarate and succinate levels[115,116] (Figure 3).

The FH and SDH enzymes responsible for the catabolism of fumarate and succinate have been implicated as tumor suppressors[117]. Genetic variants in FH or SDH complex subunits, including SDHA, SDHB, SDHC, and SDHD, have been associated with increased risk of certain cancers such as hereditary leiomyomatosis and renal cell cancer (HLRCC) [118,119] as well as paraganglioma and pheochromocytoma[120-123]. Although there is limited evidence involving genetic mutants of FH or SDH complex subunit genes in GI cancer, an unusual mutation of the FH gene was found to be associated with the development of gastric leiomyoma following cutaneous and uterine leiomyomatosis[124]. Except for loss-of-function mutations, some researchers have revealed FH and SDH complex subunit gene single nucleotide polymorphisms (SNP) in patients with HCC and CRC[125,126]. Interestingly, FH was found to be downregulated in HCC patients with portal vein thrombosis due to currently unknown underlying mechanisms[127]. However, the role of FH and SDH in GI cancer remains largely unknown. Further investigation is thus necessary.

Understanding the role of oncometabolites in GI cancer could provide valuable insights into the development of novel therapeutic targets for the treatment of these cancers. Further research should be conducted to investigate the potential roles of FH and SDH in the development and progression of GI cancer and explore the possible therapeutic targets associated with the regulation of these enzymes. By gaining a better understanding of oncometabolites in GI cancer, we may be able to develop more effective therapies and improve patient outcome.

EXPLORING BIOENERGETIC REGULATORS AS TARGETS FOR GI CANCER THERAPY

Our current understanding of metabolic reprogramming and bioenergetic alterations in cancer has led to the emergence of several potential drugs that target the bioenergetics of cancer cells, offering a promising avenue for anti-cancer therapy. These drugs can be classified into two main categories based on their mode of action: targeting aerobic glycolysis/lactate biosynthesis and transportation, or targeting the TCA cycle and coupled OXPHOS (Figure 4).

To target aerobic glycolysis, several strategies have been developed including blocking glucose import by targeting GLUT1, reducing glycolysis activity by targeting hexokinase 2 (HK2), PKMFB3, and PKM2, inhibiting lactate biosynthesis by targeting LDHA and PDK, and blocking lactate transportation through targeting MCT1/2. Targeting the TCA cycle and OXPHOS involves PDH and mitochondrial complex inhibitors. Several bioenergetic-targeted drugs have provided pre-clinical or clinical evidence in treating GI cancers. Table 4 provides a summary of these drugs. In the following sections, we will discuss the details of such strategies and the drugs used to target bioenergetic regulators during GI cancer therapy.

UNLOCKING THE POTENTIAL OF GLUCOSE METABOLISM TARGETS IN GI CANCER THERAPY

Cancer cells typically rely on increased glucose uptake, a phenomenon known as the Warburg effect, to meet energy requirements, making glucose uptake a promising target for anti-cancer therapy. As a result, GLUT1 has been identified as a potential drug target for blocking glucose uptake. Several GLUT1 inhibitors, including genistein, apigenin, fasentin, WZB117, WZB27, WZB115, STF-31, and BAY-876 have shown an ability to block glucose uptake[14]. Genistein and apigenin are natural compounds belonging to the flavonoid group, and they have been shown to inhibit hypoxia-inducible factor 1A (HIF1A) mRNA and protein expression, which leads to inactivation of downstream genes such as GLUT1 and HK2, thereby attenuating glycolysis activity[128-130]. In GI cancers, these compounds have demonstrated the ability to inhibit cancer cell proliferation, cell cycle progression, colony formation, migration, invasion, angiogenesis, stemness, spheroid formation, EMT, and to enhance cell death[131-146]. Although the majority of evidence pertaining to efficacy comes from *in vitro* cell-based assays, genistein and apigenin have entered clinical trials as a combination anti-cancer therapy for patients with CRC (NCT10985763 and NCT00609310) and PAC (NCT02336087, NCT00376948 and NCT00882765). Moreover, dietary supplementation with apigenin has been shown to significantly prevent CRC recurrence in a prospective study[147]. Fasentin, WZB117, WZB27, WZB115, STF-31, and BAY-876 are synthetic chemicals with selective activity on GLUT1 inhibition. Fasentin, WZB27, and WZB115 have shown anti-cancer potential in other pre-clinical cancer models, although there is currently little to no research on GI cancers. WZB117 has been shown to reduce glucose uptake, inhibit cell proliferation/invasion, and enhance chemosensitivity in GI cancer cell lines, as well as in xenograft models[148-151]. STF-31 has been implicated in reducing cancer stem cell stemness, cell proliferation, viability, and tumor growth in PAC and CRC cell lines, as well as in xenograft models[152,153]. BAY-876 has been found to inhibit cell proliferation, tumor growth, glucose uptake, and promote chemosensitivity in ESCA, PCA, and CRC cell lines, and in xenograft mouse models[154-156]. Although these findings are promising, WZB117, STF-31, and BAY-876 are not currently in clinical trials for GI cancer. Thus, their safety, dosage, and therapeutic response in GI cancer patients remain to be determined in future studies.

Another strategy to block glycolysis is by targeting glycolytic enzymes or attenuating glycolytic activity. A well-studied example of this strategy is the use of 2-deoxy-D-glucose (2-DG), a glucose molecule with a 2-hydroxyl group replaced by hydrogen. 2-DG is taken up by cells with high glucose uptake ability, such as cancer cells, where it serves as a competitive inhibitor of glucose[157]. Once inside the cell, 2-DG enters the glycolytic pathway and is phosphorylated by HK2 to become 2-DG-6-phosphate (2-DG-6-P), which cannot be further processed by G6P isomerase and therefore

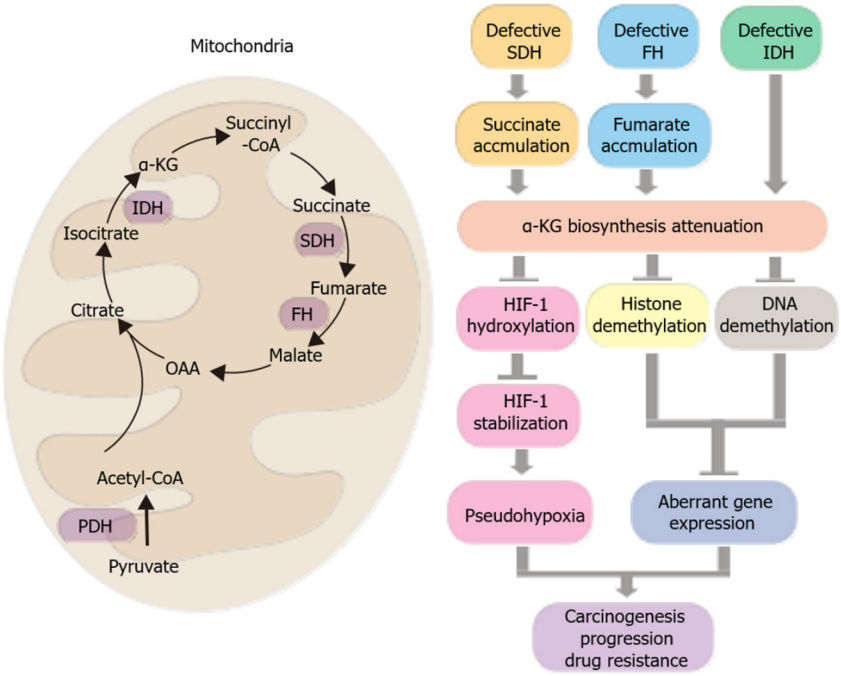

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Figure 3 Tricarboxylic acid cycle dysfunction in cancer and its role in carcinogenesis, progression, and anti-cancer drug resistance. The left panel depicts the tricarboxylic acid cycle, with succinate dehydrogenase and fumarate hydratase as key regulatory enzymes responsible for the formation of oncometabolites succinate and fumarate. The isocitrate dehydrogenase enzyme synthesizes α-ketoglutarate, which serves as a substrate for tumor suppressor pathways, such as hypoxia-inducible factor 1 hydroxylation for degradation, as well as histone and DNA demethylation. These processes can lead to pseudohypoxia and aberrant gene expression, promoting carcinogenesis, progression, and anti-cancer drug resistance. The right panel provides a summary of these relationships. OAA: Oxaloacetate; SDH: Succinate dehydrogenase; FH: Fumarate hydratase; PDH: Pyruvate dehydrogenase; IDH: Isocitrate dehydrogenase; α-KG: α-ketoglutarate; HIF-1: Hypoxia-inducible factor 1.

accumulates. Accumulated 2-DG-6-P reversely negatively inhibits HK2 activity, leading to a reduction in glycolytic activity. A derivative of 2-DG, fluorodeoxyglucose (18F-FDG), has been extensively employed in positron emission tomography (PET) to visualize the location and status of certain types of cancers[158]. In pre-clinical studies using GI cancer cell lines, as well as xenograft models and rat HCC and hamster PAC models, 2-DG has been shown to inhibit cell proliferation, tumor growth, and promote chemosensitivity[159-165]. Although 2-DG has entered clinical trials for other cancer types, only a phase I trial (NCT00096707) was conducted for patients with PAC, and the safety, dose, and efficacy of 2-DG in treating patients with other GI cancers are unknown.

Several other chemical drugs have been claimed to inhibit HK2 function, but their roles in GI cancers are unclear, with the exception of 3-bromopyruvate (3-BrPA) and lonidamine (LND). 3-BrPA is an analog of both lactate and pyruvate and shows an inhibitory effect on HK2, possibly due to its ability to induce protein alkylation[166,167]. In pre-clinical studies of GI cancers, 3-BrPA has shown its ability to inhibit cellular ATP generation, cell proliferation, tumor growth, induce mitochondrial depolarization, reduce animal serum VEGF levels, and promote cell death and chemosensitivity in GC, HCC, PAC, and CRC cell lines, as well as rabbit, transgenic mice, and xenograft mouse models[167-171]. Therapeutic efficacy and safety were only evaluated in a case report study, providing a safe and tolerable dose of 3-BrPA in patients with fibrolamellar HCC[172].

LND is an indazole derivative that was previously utilized as an anti-spermatogenic agent. In drug re-purposing studies, LND was found to have anti-cancer activity by affecting bioenergetic homeostasis, including the glycolytic pathway, through targeting HK2 *via* currently unclear mechanisms[173]. LND showed promising therapeutic efficacy by inhibiting cell proliferation, migration, invasion, cell cycle progression, and increasing chemosensitivity in HCC, CCA, and CRC cell lines, as well as in a hamster CCA model[174-179]. Encouraging results were observed in a clinical trial recruiting patients with GC, showing improved overall response rate and duration of disease progression[174]. Reversely, it was reported that administration of LND was ineffective and toxic in clinical trials recruiting patients with CRC[178, 179].

Targeting PFKFB3 is another approach to block cancer glycolysis, as it is considered an oncogene in cancers due to its high expression and role in glycolysis[180]. PFKFB3 is activated by multiple cancer-associated stimuli, including cytokines, chemokines, growth factors, and hypoxia, and then participates in glycolysis through catalyzing fructose-6-P to become F2,6BP, which can further positively enhance PFK1 activity and thus accelerate glycolysis[180]. Accordingly, PFKFB3 drugs have been identified and tested in pre-clinical and clinical studies. Among the list of candidate drugs that target PFKFB3, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFK15), and 1-pyridin-4-yl-3-[7-(trifluoromethyl)-quinolin-2-yl]-prop-2-en-1-one (PFK158) have drawn more attention than others[181]. It was found that 3PO and PFK15 inhibit cell proliferation, reduce tumor growth, attenuate angiogenesis, prevent fibrogenesis, and increase cell death in pre-clinical studies using GI cancer cell lines, transgenic mice,

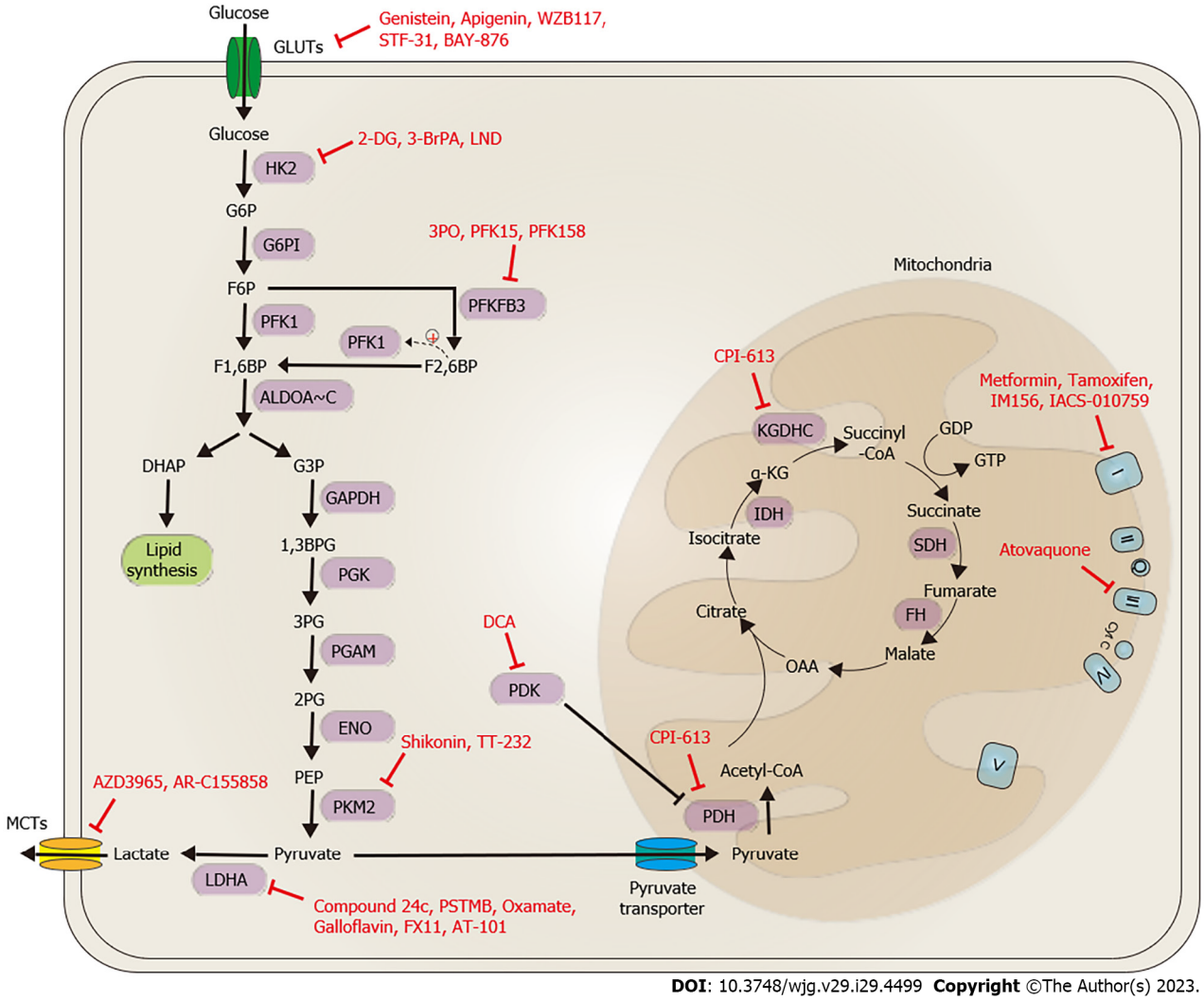

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Figure 4 Potent bioenergetic-targeting drugs for gastrointestinal cancers. Promising bioenergetic drugs for gastrointestinal cancers can be classified into two main categories based on their mode of action. The first category involves targeting aerobic glycolysis and lactate biosynthesis/transportation, while the second category involves targeting the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). Strategies to target aerobic glycolysis include blocking glucose importation through the targeting of glucose transporter 1 with compounds such as genistein, apigenin, WZB117, STF-31, and BAY-876, reducing glycolysis activity by targeting HK2 with compounds such as 2-DG, 3-BrPA, and LND, and targeting PKMFB3 and PKM2 with compounds such as 3PO, PFK15, PFK158, shikonin, and TT-232. Lactate biosynthesis can be inhibited by targeting LDHA with compounds such as compound 24c, PSTMB, oxamate, galloflavin, FX11, and AT-101, and PDK with DCA. Lactate transportation can be blocked by targeting MCT1/2 with compounds such as AZD3965 and AR-C155858. Targeting the TCA cycle and OXPHOS involves using inhibitors of pyruvate dehydrogenase, such as CPI-613, and mitochondrial complex I with metformin, tamoxifen, IM156, IACS-010759, and complex III with atovaquone. GLUT: Glucose transporter; HK: Hexokinase; G6P: Glucose-6-phosphate; G6PI: Glucose-6-phosphate isomerase; F6P: Fructose-6-phosphate; NADPH: Nicotinamide adenine dinucleotide phosphate; PFK1: Phosphofructokinase-1; F2,6BP: Fructose-2,6-bisphosphate; PFKBP3: Fructose-2,6-biphosphatase 3; F1,6BP: Fructose-1,6-bisphosphate; G3P: Glyceraldehyde-3-phosphate; DHAP: Dihydroxyacetone phosphate; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; 1,3BPG: 1,3-bisphosphoglycerate; 3PG: 3-phosphoglycerate; PGK: Phosphoglycerate kinase; PGAM: Phosphoglycerate mutase; 2PG: 2-phosphoglycerate; ENO: Enolase; PEP: Phosphoenolpyruvate; PKM1/2: Pyruvate kinase isozyme M1/M2; LDH: Lactate dehydrogenase; MCT: Monocarboxylate transporter family; PDH: Pyruvate dehydrogenase; IDH: Isocitrate dehydrogenase; α-KG: α-ketoglutarate; OAA: Oxaloacetate; SDH: Succinate dehydrogenase; FH: Fumarate hydratase; I: Mitochondrial complex I; II: Mitochondrial complex II; III: Mitochondrial complex III; IV: Mitochondrial complex IV; V: Mitochondrial complex V; Q: Co-enzyme Q; cyto C: Cytochrome *c*; KGDHC: α-ketoglutarate dehydrogenase complex.

xenograft mouse models, and HCC rat models[182-189]. Intriguingly, it was also found that 3PO suppresses glucose uptake *via* a 14C-2-DG tracing system[184]. Although there is no pre-clinical evidence of efficacy in GI cancers, the safety, tolerated dose, and therapeutic efficacy of PKF158 have been evaluated in a Phase I clinical trial (NCT02044861) that involved patients with solid tumors[190].

One strategy proposed to inhibit glycolysis activity is to target the last enzyme in the glycolytic pathway –PKM2. PKM2 targeting is based on its glycolysis role as well as aberrant expression in cancer-associated events[191]. While many drugs have shown the ability to inhibit PKM activity, only two, TT-232 and Shikonin, have been confirmed efficacious in pre-clinical studies. Both TT-232 and Shikonin have been found to inhibit GI cancer cell proliferation, migration, invasion, cell cycle progression, and tumor growth, as well as enhance cell death[192-200]. However, the efficacy of these drugs in treating GI cancers is still unclear and requires further investigation. Both drugs have entered clinical trials for specific cancers, showing promise as cancer therapy targets.

Table 4 Promising novel bioenergetics targeting drugs for gastrointestinal cancer therapy

Inhibitor	Target	GI model	Consequence	Clinical trial	Ref.
Targeting glucose transportation					
Genistein	HIF1A, GLUT1 and HK2	GC, ESCA, HCC, CCA, PCA, and CRC cell lines	Inhibited cancer cell proliferation, cell cycle progression, migration, invasion, angiogenesis, stemness, spheroid formation, EMT, and promoted apoptosis	CRC patient, phase I/II (NCT10985763), and PAC patient, phase I/II (NCT02336087, NCT00376948 and NCT00882765)	[131-140]
Apigenin	HIF1A, GLUT1 and HK2	GC, ESCA, HCC, CCA, PCA, and CRC cell lines	Inhibited cancer cell proliferation, colony-forming, cell cycle progression, migration, invasion, angiogenesis, and induced apoptosis	CRC patient, phase II (NCT00609310)	[141-146]
WZB117	GLUT1	HCC, CCA, PAC, and CRC cell lines, and xenograft models	Reduced glucose uptake, inhibits cell proliferation, and invasion, and enhanced chemosensitivity	None in GI cancers	[148-151]
STF-31	GLUT1	PAC and CRC cell lines, and xenograft model	Reduced cancer stem cell properties, such as stemness, and inhibits cell proliferation, viability, and tumor growth	None in GI cancers	[152, 153]
BAY-876	GLUT1	ESCA, PCA, and CRC cell lines, and xenograft mouse models	Reduced cancer cell proliferation, tumor growth, and glucose uptake, while also increased chemosensitivity	None in GI cancers	[154-156]
Targeting glucose metabolism					
2-Deoxy-D-glucose (2-DG)	HK2	GC, ESCA, HCC, PAC and CRC cell lines, xenograft models, and rat HCC and hamster PAC models	Inhibited cell proliferation, tumor growth, and promoted chemosensitivity	PAC patient, phase I (NCT00096707)	[159-165]
3-Bromopyruvate (3-BrPA)	HK2	GC, HCC, PCA, and CRC cell lines, and rabbit, transgenic mouse and xenograft mouse models	Inhibited cellular ATP generation, cell proliferation, and tumor growth. Also induced mitochondrial depolarization, reduced animal serum VEGF levels, and promoted cell death and chemosensitivity	HCC patient, case report[170]	[167-170]
Lonidamine (LND)	HK2	HCC, CCA, and CRC cell lines, hamster CCA model, and GC and CRC patients	Inhibited cell proliferation, migration, invasion, and cell cycle progression. Increased chemosensitivity, patient overall response rate, and duration of disease progression in GC patients. However, was ineffective and toxic in advanced CRC patients	GC patient, phase II[172], CRC patients, phase II[176,177]	[174-179]
3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO)	PFKFB3	HCC, PAC, and CRC cell lines, and transgenic and xenograft mouse models	Inhibited glucose uptake, cell proliferation, tumor growth, angiogenesis, fibrogenesis, and promoted cell death	None in GI cancers	[182-184]
1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFK15)	PFKFB3	GC, HCC, PAC, and CRC cell lines, xenograft models, and HCC rat model	Inhibited cell proliferation, migration, invasion, cell cycle progression, tumor growth, and enhanced cell death	None in GI cancers	[185-189]
1-pyridin-4-yl-3-[7-(trifluoromethyl)-quinolin-2-yl]-prop-2-en-1-one (PFK158)	PFKFB3	None in GI cancers	None in GI cancers	Solid tumor patients, phase I (NCT02044861)	[190]
Shikonin	PKM2	GC, ESCA, HCC, CCA, PCA, and CRC cell lines, and xenograft mouse models	Inhibited cell proliferation, migration, invasion, cell cycle progression, tumor growth, and enhanced cell death	None in GI cancers	[192-197]
TT-232	PKM2	HCC, PAC, and CRC cell lines,	Inhibited cell proliferation, tumor growth, and enhanced cell death	None in GI cancers	[198-200]

		and xenograft mouse models			
Targeting lactate biosynthesis					
Dichloroacetate (DCA)	PDK	GC, ESCA, HCC, PAC, and CRC cell lines, xenograft models, and B6C3F1 mice	Reduced lactate production, cell proliferation, migration, and increased chemosensitivity. Showed synergistic anticancer effects in HCC. However, promoted hepatocarcinogenesis in B6C3F1 mice	CRC patient, phase I (NCT00566410)	[203-207]
Compound 24c	LDHA	PAC cell lines, and xenograft model	Suppressed cell proliferation, colony formation, enhanced cell apoptosis, arrested cell at G2 phase, repressed xenograft growth, and re-programmed cancer metabolism, with minimal impact on mouse weight	None in GI cancers	[210]
1-(Phenylseleno)-4-(Trifluoromethyl) Benzene (PSTMB)	LDHA	HCC and CRC cell lines	Inhibited cell proliferation, reduced cell viability, attenuated LDHA activity, lowered lactate levels, and induced mitochondria-mediated apoptosis	None in GI cancers	[211]
Oxamate	LDHA	GC, ESCA, HCC, PCA, and CRC cell lines	Suppressed LDHA activity, lactate production, cell proliferation, migration, MMP9 expression, pro-inflammatory cytokines, EMT transition, and AKT/ERK/mTOR signaling pathways, while enhanced apoptosis, senescence, protective autophagy, and metabolic rewiring	None in GI cancers	[212-218]
Galloflavin	LDHA	HCC, PCA, and CRC cell lines	Reduced ATPase activity and expression levels of heat shock proteins, inhibited cell proliferation, lactate production, pro-inflammatory cytokines, and EMT transition, while promoting apoptosis and senescence	None in GI cancers	[215, 218-220]
FX11	LDHA	HCC, PCA, and CRC cell lines, and xenograft mouse models	FX11 reduced lactate production and ATP levels, suppressed cell proliferation, migration, invasion, and xenograft tumor growth, while enhancing apoptosis. However, in a PCA patient-derived mouse xenograft model, FX11 was only effective in attenuating tumor growth in the presence of mutant TP53	None in GI cancers	[221-225]
Gossypol (AT-101) or its derivatives	LDHA	GC, ESCA, HCC, PAC and CRC cell lines, GC and xenograft mouse models, and ESCA patient	Reduced cell viability, suppressed cell proliferation, migration, and tumor growth, down-regulated cancer stem cell markers CD133, Nanog, LC3, and YAP-1, enhanced apoptosis, protective autophagy. and complete response rate/prognosis	ESCA patient, phase I/II (NCT00561197)	[226-240]
Targeting lactate transportation					
AZD3965	MCT1/2	GC, ESCA, HCC, CRC cell lines	Inhibited cell proliferation and tumor growth, while increasing intracellular lactate concentration, TCA-related metabolites, mitochondrial metabolism, and chemosensitivity. Also decreased intracellular pH	None in GI cancers	[242-246]
AR-C155858	MCT1/2	GC, PAC, and CRC cell lines, and xenograft mouse models	Inhibited cell proliferation, spheroid forming ability, and tumor growth, while decreased glycolysis and increased intracellular lactate concentration, TCA-related metabolites, mitochondrial metabolism, and chemosensitivity	None in GI cancers	[247-249]
Targeting mitochondrial OXPHOS					
Metformin	Mitochondrial complex I	GC, ESCA, HCC, CCA, PAC, and CRC cell lines, xenograft models, and ESCA, HCC, CCA, PCA and CRC patients	Suppressed cell proliferation, migration, cell cycle progression, and tumor growth while increasing chemosensitivity and cell death. Also re-programmed the tumor immune microenvironment in ESCA patients	ESCA patient, phase II (ChiCTR-ICR-15005940), HCC patient, phase I (CTRI/2018/07/014865), CCA patient, phase Ib (NCT0249674), PCA patient, phase II (NCT01210911 and NCT01167738), and CRC patient, phase II (NCT01312467, NCT03047837, and NCT01941953)	[252-265]

Tamoxifen	Mitochondrial complex I	GC, ESCA, HCC, CCA, PAC and CRC cell lines, CRC murine model, and ESCA, HCC and PAC patients	Inhibited cell proliferation, tumor growth, metastasis, and increased chemosensitivity. However, no prolonged survival benefits have been observed in HCC patients, and in some cases, there may even be a higher risk of death	ESCA patient, phase I (NCT02513849), PAC patient, phase II[272-274], and HCC patient, phase III (NCT00003424)	[267-273, 277]
IM156	Mitochondrial complex I	GC and CRC patients	Considered tolerable in human subjects, with stable disease being the most common response. Combinatorial therapy may be necessary for improved efficacy	GC and CRC patients, phase I (NCT03272256), and PAC patient, phase Ib (NCT05497778)	[278]
IACS-010759	Mitochondrial complex I	PAC cell lines, and CCA, PAC, and CRC patients	Reduced cell viability and generally well tolerated, but may induce neurotoxicity, peripheral neuropathy, and behavioral/physiological changes in mice. Increased blood lactate levels	CCA, PAC, and CRC patient, phase I (NCT03291938)	[279, 280]
Atovaquone	Mitochondrial complex III	GC, HCC, PAC and CRC cell lines, and xenograft models	Reduced OXPHOS, oxygen consumption rate, cell viability, cell proliferation, and cell cycle progression. Inhibited tumor growth and enhanced cell death	None in GI cancers	[283-285]
Targeting TCA cycle					
CPI-613	PDH and KGDHC	GC, ESCA, PAC and CRC cell lines, xenograft mouse models, and GC mouse model	Inhibited cell proliferation, cell viability, tumor growth, and metastasis, while increased cell death and chemosensitivity. In PAC patients, also increased the overall response rate	PAC patient, phase I (NCT01835041) and III (NCT03504423), HCC and CCA patients, phase I/II (NCT01766219), and CRC patients, phase I (NCT05070104 and NCT02232152)	[287-291]

GI: Gastrointestinal; ESCA: Esophageal cancer; GC: Gastric cancer; HCC: Hepatocellular carcinoma; CCA: Cholangiocarcinoma; PAC: Pancreatic cancer; CRC: Colorectal cancer; LDHA: Lactate dehydrogenase subunit A; MCT1/2: Monocarboxylate transporter family 1/2; HIF1A: Hypoxia inducible factor 1A; GLUT1: Glucose transporter 1; HK2: Hexokinase 2; PFKFB3: Fructose-2,6-biphosphatase 3; PKM2: Pyruvate kinase isozyme M2; PDK: Pyruvate dehydrogenase kinase; PDH: Pyruvate dehydrogenase; KGDHC: Alpha-ketoglutarate dehydrogenase complex; EMT: Epithelial-mesenchymal transition; OXPHOS: Oxidative phosphorylation.

EXPLORING LACTATE BIOSYNTHESIS AND TRANSPORT AS A POTENTIAL STRATEGY FOR GI CANCER THERAPY

As mentioned above, the Warburg effect is a common phenomenon in many cancers for which glycolysis is upregulated even in the presence of oxygen. This results in the accumulation of lactate, which is the last product of glycolysis. The PDK class of enzymes play a key role in deciding whether pyruvate is converted to lactate or enters the TCA cycle. Under hypoxia, PDKs are transcriptionally upregulated by HIF1A in cancers, promoting the inactivation of PDH through PDK-mediated phosphorylation. This leads to elevated lactate biosynthesis, resulting in excessive lactate levels that can promote carcinogenesis or progression[201]. Therefore, targeting PDKs is a potential strategy to inhibit lactate synthesis. Although several candidate drugs that target PDKs have been proposed, dichloroacetate (DCA) has been the most convincing inactivator of PDKs[202]. DCA has been shown in numerous pre-clinical studies on GI cancer to reduce lactate production, cell proliferation, migration, and increase chemosensitivity[203-207]. It has also shown synergistic anti-cancer activity in HCC despite concerns that it may promote hepatic carcinogenesis in B6C3F1 mice[205,208]. Despite promising pre-clinical results, clinical studies are still necessary to determine the efficacy and safety of DCA during cancer therapy. A clinical trial recruiting patients with CRC has been conducted to evaluate DCA as a potential anti-cancer drug (NCT00566410).

In previous studies on lactic acid inhibitors for anti-cancer therapy, the focus has been on inhibiting the enzymes responsible for lactate biosynthesis, namely LDH. TLDH complex composition has been investigated as a crucial factor in determining the fate of lactate biosynthesis or catabolism, and LDHA homo-tetramer (LDH5 or A4) has been considered the most effective complex for lactate biosynthesis. Accordingly, the currently established strategy is to identify LDH inhibitors with high selectivity against LDHA[209]. Although many candidates exist, including small peptides, small interfering RNAs (siRNAs), small chemical molecules, and natural compounds, only a few have progressed towards clinical use in anti-cancer therapy. Compound 24c and 1-(Phenylseleno)-4-(Trifluoromethyl) Benzene (PSTMB) are small compounds that have recently been identified as capable of selectively inhibiting LDHA, suppressing cancer cell aggressiveness, and enhancing cell death in both PCA cells and xenograft mouse models[210] as well as HCC and CRC cells [211]. Notably, Compound 24c has little effect on mouse weight, perhaps due to its relatively strong activity to reprogram metabolic profiling[210]. In contrast, oxamate, galloflavin, and FX11 have a longer history than Compound 24c and PSTMB in targeting LDHA. Pre-clinical evidence shows promise in suppressing GI cancer cell aggressiveness by targeting LDHA and other cancer-associated signaling pathways, suggesting possible treatment of GI cancers[212-225]. Despite this evidence, there is still a lack of clinical results to support the safety and efficacy of these LDHA-targeting drugs in GI cancer patients. An early natural compound, gossypol (AT-101), derived from the cotton plant, is one exception. Gossypol

and its derivatives have proven potent inhibitors of LDHA[226]. Gossypol not only reduces the aggressiveness of GI and other cancers, but also has a strong cytotoxic effect on cancer cells[226-240]. Most importantly, gossypol has entered a phase I/II clinical trial (NCT00561197) to evaluate its safety and efficacy in treating patients with esophageal cancer, showing significant improvement in complete response and survival rates[231]. Therefore, gossypol may be the most promising clinical drug targeting LDHA to date for use in GI cancers.

Excessive intracellular accumulation of lactate is a hallmark of many cancer types, which necessitates MCTs in transporting lactate from highly glycolytic cancer cells. Secretory lactate can acidify the extracellular microenvironment, which can impact the tumor microenvironment[241]. While secretory lactate was initially considered a waste product of cancer cells, recent evidence has suggested that it serves as an alternative fuel for oxidative cancer cells, leading to enhanced aggressiveness[56]. Therefore, MCT targets have emerged as an alternative strategy for anti-cancer therapy [241]. Among the various compounds proposed to target MCTs in cancer, AZD3965 and AR-C155858 have received more attention from researchers. Both drugs have demonstrated potential in targeting MCTs, inhibiting GI cancer cell aggressiveness, and stunting tumor growth both *in vitro* and *in vivo*[242-249]. While AZD3965 has entered the clinical trial phase, further investigation is needed to determine the safety and therapeutic efficacy of these drugs in patients with GI cancer. Notably, the development of MCT inhibitors has faced several challenges, including the presence of MCT isoforms and the need for inhibitors that selectively target cancer cells without affecting normal tissues[58,250]. In this regard, approaches and strategies to develop selective MCT inhibitors are being actively pursued. While MCT inhibitors hold promise as a potential anti-cancer therapy, further research is needed to fully understand their mechanisms of action and optimize their clinical applications.

TARGETING OXPHOS AS A POTENTIAL THERAPEUTIC STRATEGY FOR GI CANCER

Excessive OXPHOS activity has been observed in certain cancers and has been associated with more aggressive phenotypes/unfavorable clinical outcomes, making it a novel target for anti-cancer therapy[251]. Attenuating OXPHOS activity has been proposed as the best strategy to target OXPHOS, leading to the identification of a large number of candidate compounds that target mitochondrial complex I. Metformin, a compound that has long been used to treat diabetes, has been reported to exhibit mitochondrial complex I inhibition activity and can impact cancer cell aggressiveness/tumor growth in both GI cancer cell lines and xenograft models[252-265]. Metformin has advanced to clinical trials in combination with other anti-cancer regimens for patients with GI cancers, such as ESCA patients in Phase II (ChiCTR-ICR-15005940), HCC patients in Phase I (CTRI/2018/07/014865), CCA patients in Phase Ib (NCT0249674), PCA patients in Phase II (NCT01210911 and NCT01167738), and CRC patients in Phase II (NCT01312467, NCT03047837, and NCT01941953). It was found that metformin combination therapy can provide benefit to patients, perhaps through reprogramming the tumor immune microenvironment[258].

Recent studies have proposed several candidates as mitochondrial complex I-targeting compounds in addition to metformin. Among them, tamoxifen, IM156, and IACS-010759 have gained attention as potential anti-cancer agents. Tamoxifen is an anti-estrogen agent that has been clinically used to treat breast cancer patients with positive estrogen-receptor (ER) expression[266]. Interestingly, tamoxifen has also been shown to inhibit cancer cell aggressiveness, tumor growth, metastasis, and increase chemosensitivity in GI cancers[267-273]. This effect is thought to be through an ER-independent anti-cancer pathway[269]. Tamoxifen has been used as a monotherapy or combined therapy in several clinical trials, including an early phase trial in ESCA patients, Phase II trials in PAC patients[274-276], and a Phase III trial in HCC patients (NCT00003424). Tamoxifen has been found to be tolerable, safe, and with manageable adverse effects, while a Phase III trial in HCC patients found that tamoxifen monotherapy either offered no effect or decreased survival in patients with unresectable HCC[277]. This result has slowed the advancement of tamoxifen in GI cancers and requires further investigation.

IM156 and IACS-010759 are two novel mitochondria-targeting drugs that specifically inhibit mitochondrial complex I. While both compounds have shown promising results in pre-clinical studies against certain cancer cell lines, their potential in treating GI cancers involves limited evidence. Interestingly, IM156 has entered Phase I clinical trials in patients with GC, CRC and PCA (NCT03272256 and Janku *et al*[278]), demonstrating tolerability and safety. However, IM156 monotherapy in patients with GC and CRC offered only disease stabilization, indicating the need for further study.

On the other hand, IACS-010759 has shown significant cell viability reduction in PCA cell lines[279], leading to the initiation of a Phase I clinical trial (NCT03291938) to evaluate clinical efficacy and safety in patients with solid tumors due to CCA, PAC, and CRC. However, a recent publication reported that although IACS-010759 was tolerable and safe, it increased blood lactate levels and neurotoxicity while offering only limited anti-cancer efficacy. A reverse translational study using mice also found IACS-010759 to induce behavioral and physiological changes indicative of peripheral neuropathy, minimizing the possibility of combined therapy with specific anti-cancer compounds (*e.g.*, histone deacetylase 6 inhibitor). The development of mitochondrial complex I inhibitors is ongoing[280].

While the mitochondrial complex I inhibitors metformin, tamoxifen, IM156, and IACS-010759 hold promise as potential treatments for GI cancer, further studies are needed to evaluate their efficacy and safety, particularly in combination with other anti-cancer compounds. The development of more selective and potent mitochondrial complex I inhibitors may help overcome side effects and improve efficacy in cancer treatment.

The targeting of mitochondrial complexes other than complex I has also been proposed as a strategy for anti-cancer therapy[281]. One such compound of note is atovaquone, which was identified as a mitochondrial complex III inhibitor during a drug re-purposing study[282]. Pre-clinical studies have evaluated the potential of atovaquone as an anti-cancer agent in GI cancer cell lines and xenograft models, and have shown its ability to reduce OXPHOS, OCR, cell viability, cell

proliferation, cell cycle progression, and tumor growth, while enhancing cell death[283-285]. Despite promising results, atovaquone is currently in clinical trials for patients with non-small cell lung cancer (NCT04648033) and acute myeloid leukemia (NCT03568994) but not for patients with GI cancer. Further studies are needed to determine drug tolerability, safety, and therapeutic efficacy in patients with GI cancer. Nonetheless, the potential benefits of targeting OXPHOS make for a promising strategy in GI cancer therapy. However, the potential toxicity of these inhibitors in normal cells must be carefully evaluated before being considered as viable anti-cancer agents. In addition, the development of resistance to mitochondrial inhibitors, similar to the resistance seen with other anti-cancer agents, highlights the need for combination therapy.

POTENTIAL OF TCA CYCLE TARGETS IN GI CANCER THERAPY

The TCA cycle is a critical metabolic pathway that fuels bioenergetic processes in cells. Targeting the TCA cycle has emerged as a potential strategy for anti-cancer therapy[286]. Various agents have been tested for their anti-cancer efficacy, including AGI-5195, AG-221, AG-881, and CPI-613[286]. Among these compounds, CPI-613 is the only PDH and alpha-ketoglutarate dehydrogenase complex (KGDHC) dual targeting agent that has shown promising anti-cancer properties in GI cancer models both *in vitro* and *in vivo*[287-291]. The tolerability and safety of CPI-613, alone or in combination with other agents, has been evaluated or is currently being studied in patients with HCC, CCA, and CRC (NCT01766219, NCT05070104 and NCT02232152). However, a recent Phase III trial (NCT03504423) evaluating the anti-cancer efficacy of CPI-613 in patients with advanced PAC failed to improve survival rate but improved overall response rate[292]. This outcome is disappointing, combining CPI-613 with other drugs such as gemcitabine or nab-paclitaxel may provide better results.

The TCA cycle is a complex pathway, and there are multiple enzymes and metabolites that could be targeted for anti-cancer therapy. For example, the isocitrate dehydrogenase 1 and 2 (IDH1/2) enzymes play a crucial role in the TCA cycle, and mutations in these enzymes have been observed in several types of cancer, including gliomas and acute myeloid leukemia (AML)[293]. Enasidenib and ivosidenib are two IDH1/2 inhibitors that have been approved for the treatment of relapsed or refractory AML[294,295]. In GI cancers, however, the efficacy of IDH1/2 inhibitors is still under investigation [296]. In addition to IDH1/2 inhibitors, other TCA cycle inhibitors are being explored for anti-cancer therapy. For example, IDH1/2 mutant tumors are sensitive to glutaminase inhibitor CB-839, which targets glutamine metabolism [297]. Another TCA cycle inhibitor, BPTES, has shown anti-cancer efficacy in pre-clinical studies by blocking the activity of the glutaminase enzyme[298]. However, our understanding of these inhibitors in GI cancer treatment is still limited.

Targeting the TCA cycle and associated bioenergetic processes is a promising approach for anti-cancer therapy. While CPI-613 has shown some success in GI cancer models, the failure in Phase III trial underscores the need for continued research and combination therapy. Other TCA cycle inhibitors, such as IDH1/2 and glutaminase inhibitors, are being evaluated for their anti-cancer efficacy in GI cancers, offering hope for future treatments.

DISCUSSION AND FUTURE PERSPECTIVE

Cancer cells undergo significant metabolic changes which involve alteration to the nuclear and mitochondrial genomes as well as cell microenvironment. Understanding the molecular mechanisms behind these alterations is critical for the development of effective cancer therapies. Next-generation technologies such as metabolic profiling, single-cell sequencing, and metabolic tracing can provide insights into the regulation of mitochondrial metabolism in different cancer types. However, developing therapies based on altered metabolism is challenging due to the diverse metabolic patterns observed across different cancer cells.

Simply targeting a single bioenergetic enzyme or pathway may not be enough to effectively inhibit cancer cell growth, as metabolic symbiosis enables cancer cells to adapt to harsh tumor environments. One potential strategy is to treat the metabolic patterns of different cellular subpopulations in the tumor microenvironment to create a homogeneous metabolic population for targeting.

Bioenergetic enzymes have been explored as a way to inhibit cancer cell growth, with some small-molecule inhibitors of glucose metabolism showing significant inhibition in various cancers. However, clinical translation of these inhibitors has been limited by side effects. Other small-molecule inhibitors and natural products that regulate key bioenergy enzymes have also shown promise, but their specific mechanisms and targets require further investigation. Developing anticancer drugs targeting bioenergetic enzymes remains a significant challenge due to the unique metabolic features of cancer cells. Targeted drugs have shown anticancer effects in various tumor models, and combining them with conventional anticancer drugs is a promising strategy.

High-throughput multi-omics and spatial omics can help elucidate the heterogeneity of cancer cells and provide opportunities for therapeutic drugs targeting the bioenergetics of malignant tumors. Unbiased CRISPR-Cas9 synthetic lethality screening of metabolic genes that favor anti-cancer responses, particularly *in vivo*, could provide an avenue towards the identification of bioenergetic targets of interest. The ultimate goal is to develop drugs that simultaneously disable cancer cells while synergizing with targeted therapies.

However, while targeting bioenergetic pathways in cancer cells shows promise, it also has the potential to affect normal cells and tissues that rely on these pathways. Therefore, careful consideration and further research are needed to ensure that therapies targeting bioenergetics in cancer cells are specific and effective while minimizing potential side effects on normal cells and tissues. Additionally, combination therapies that target multiple pathways may be necessary

to achieve optimal therapeutic effects.

CONCLUSION

The metabolic reprogramming and bioenergetic alteration of cancer cells, particularly their utilization of glucose fermentation (the Warburg effect) for energy production, are well-known phenomena. However, comprehensive summaries of these alterations and their oncogenetic links in GI cancers are lacking. This review provides a summary of the interplay between aerobic glycolysis, the TCA cycle, and OXPHOS in cancer cells, including the molecular mechanisms that trigger these alterations. It also explores the role of HIFs, tumor suppressors, and the oncogenetic link between hypoxia-related enzymes, bioenergetic changes, and GI cancer. Additionally, this review details various anti-cancer drugs and strategies for treating GI cancers, along with the challenges associated with them. Understanding dysregulated cancer cell bioenergetics is critical for effective treatments, although the diverse metabolic patterns present challenges for targeted therapies. Further research is needed to comprehensively understand the specific mechanisms of inhibiting bioenergetic enzymes, address side effects, and utilize high-throughput multi-omics and spatial omics for insights into the heterogeneity of GI cancer cells in targeted bioenergetic therapies.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Antagonizing adipose tissue-derived exosome miR-103-hepatocyte phosphatase and tensin homolog pathway alleviates autophagy in non-alcoholic steatohepatitis: A trans-cellular crosstalk

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Abstract

BACKGROUND

Obesity plays a vital role in the occurrence and development of non-alcoholic steatohepatitis (NASH). However, the underlining mechanism is still unclear, where adipose tissue (AT) derived exosomes may actively participate. MicroRNAs (miRNAs) are commonly secreted from exosomes for cell communication. Though the regulation of miR-103 on insulin sensitivity has been reported, the specific role of AT-derived exosomes miR-103 in NASH is still vague and further investigation may provide novel therapeutic choices.

AIM

To determine the specific role of AT-derived exosomes miR-103 in developing NASH through various methods.

METHODS

The expression levels of miR-103 in the AT-derived exosomes and livers were detected and compared between NASH mice and control. The effect of miR-103

on NASH progression was also explored by antagonizing miR-103, including steatosis and inflammation degree changes. The interaction between miR-103 and the autophagy-related gene phosphatase and tensin homolog (PTEN) was confirmed by dual-luciferase reporter assay. The role of the interaction between miR-103 and PTEN on autophagy was verified in NASH-like cells. Finally, the effects of miR-103 from adipose-derived exosomes on NASH and autophagy were analyzed through animal experiments.

RESULTS

The expression of miR-103 was increased in NASH mice, compared to the control, and inhibition of miR-103 could alleviate NASH. The results of the dual-luciferase reporter assay showed miR-103 could interact with PTEN. MiR-103-anta decreased p-AMPKa, p-mammalian target of rapamycin (mTOR), and p62 but increased the protein levels of PTEN and LC3-II/I and the number of autophagosomes in NASH mice. Similar results were also observed in NASH-like cells, and further experiments showed PTEN silencing inhibited the effect of miR-103-anta. AT derived-exosome miR-103 aggravated NASH and increased the expressions of p-AMPKa, p-mTOR, and p62 but decreased the protein levels of PTEN and LC3-II/I and the number of autophagosomes in mice.

CONCLUSION

AT derived-exosome increased the levels of miR-103 in the liver, and miR-103 aggravated NASH. Mechanically, miR-103 could interact with PTEN and inhibit autophagy.

Key Words: Non-alcoholic steatohepatitis; Nonalcoholic fatty liver disease; Exosomes; Phosphatase and tensin homolog

Core Tip: Our study confirms the important role of miR-103-phosphatase and tensin homolog-autophagy axis in the pathogenesis of non-alcoholic steatohepatitis (NASH). More importantly, the elevation of miR-103 in the liver of NASH mice is partly due to adipose tissue exosome secretion and integration, which also partially explains the mechanism of obesity leading to NASH.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is recognized as the hepatocellular manifestation of metabolic syndrome, characterized by hepatic lipid accumulation and inflammation, and precluded with secondary causes, such as chronic viral hepatitis, significant alcohol consumption, long-term use of steatogenic medication, and other chronic liver diseases including autoimmune hepatitis, hemochromatosis, Wilson's disease[1]. NAFLD is categorized into NAFL, non-alcoholic steatohepatitis (NASH), fibrosis, and even cirrhosis according to histological changes in different disease stages. Among them, NASH is considered the watershed in NAFLD and is defined as the presence of 5% hepatic steatosis and ensuing hepatocyte injury[1]. According to a previous meta-analysis, the pooled overall global prevalence of NAFLD was estimated to be 25.24% [95% confidence interval (CI): 22.10-28.65], while the pooled overall NASH prevalence among biopsied NAFLD patients was 59.10% (95%CI: 47.55-69.73)[2]. Moreover, fibrosis, which is closely related to liver cirrhosis, liver cancer, and other end-stage liver diseases, is more likely to occur in NASH patients than NAFL patients [3]. Those findings support the importance of NASH and suggest that the burden of disease caused by NASH needs to be paid adequate attention. NASH has become one of the leading causes of cirrhosis and the second leading cause of liver transplantation in the United States[4,5].

NAFLD is commonly associated with metabolic comorbidities such as obesity, diabetes mellitus, and dyslipidemia[2,6]. The prevalence of obesity is as high as 51.34% (95%CI: 41.38-61.20) and 81.83% (95%CI: 55.16-94.28) among NAFLD and NASH patients, respectively[2]. The effect of obesity on NAFLD has been intensively explored. On the one hand, the expansion of adipose tissue (AT) in obese people leads to increased circulating free fatty acids (FFAs) and leptin and decreased adiponectin, which leads to intrahepatic fat accumulation. On the other hand, the chronic inflammatory state caused by obesity will further lead to the infiltration of inflammatory cells in the liver, resulting in the progression of NAFLD[7]. Due to the important role of obesity in the occurrence and development of NAFLD, in-depth research on the mechanism of obesity leading to NASH may provide new therapeutic targets.

Exosomes are extracellular vesicles secreted by various cells and serve as an essential means of intercellular communication by delivering microRNAs (miRNAs), bioactive lipids, and regulatory proteins from one cell to another [8]. Previous studies have shown that AT-derived exosomes are essential in regulating insulin sensitivity[9,10], a common

manifestation of metabolic syndrome in patients with NAFLD. Previous studies have also shown that AT-derived exosome miRNAs are involved in the occurrence and development of various metabolic-related diseases[11]. Among them, miR-103 attracted our attention since previous studies have shown that miR-103 is involved in regulating insulin sensitivity[12]. The specific role of AT-derived exosomes in the development of NASH also deserves further study. Therefore, in this study, we focused on the specific role of AT-derived exosomes miR-103 in developing NASH through various methods.

MATERIALS AND METHODS

Ethics statement

This study followed the guidelines for the Care and Use of Laboratory Animals of the National Institute of Health. The animal protocol was approved by the institutional review board of the Tab of Animal Experimental Ethical Inspection of the First Affiliated Hospital of Zhejiang University. The Reference Number is 2020-1407.

The successful construction of NASH animal and cell models

C57BL/6 mice were routinely fed a high-fat diet for 12 wk to establish the NASH animal model. According to different treatments, they were initially divided into the control group (12% kcal fat, 66% kcal carbohydrate, 22% kcal protein) and the model group (60% kcal fat, 20% kcal carbohydrate, 20% kcal protein)[13]. Starting from the 13th wk, 40 mg/kg miR-negative control (NC)-anta and miR-103-anta were injected into the mice from the model group (dissolved in 0.2 mL normal saline) through the tail vein every 2 d thrice to construct miR-NC-anta model group and miR-103-anta model group. The control and model groups were injected with blank normal saline thrice (n = 10 in each group). Finally, the mice were sacrificed, where liver tissue, abdominal AT, and serum were collected and stored in a cryostorage tube at -80 °C for further analysis.

NASH-like cell model was constructed by conventional oleic acid (OA)-palmitic acid (PA) mixture culture (OA:PA = 2:1)[14]. Firstly, 128.2 mg PA (molecular weight: 256.42) was sequentially retrieved on a precision balance, 1 mL 1 M OA was added, vortex dissolved, and mixed in a small whirlpool, and then completely dissolved in a water bath at 55 °C-65 °C to obtain 1.5 M FFA mixture. After that, 1.5 M FFA with DMSO was dissolved into 0.1 M working solution. In the model group, Alpha mouse liver 12 (AML-12) cells were added with 400 μM FFA and cultured for 24 h, followed by transfection with miR-103-anta and its sh-phosphatase and tensin homolog (PTEN) and their relative controls. All further *in vitro* experiments were performed on those cells.

Exosome isolation and confirmation

Exosomes were extracted from the filtrate according to the manufacturer's instructions[15]. The diluted exosomes were subjected to NanoFCM (China) for transmission electron microscopy (TEM) and size distribution analysis for further confirmation.

Quantitative real-time polymerase chain reaction, ELISA, and western blotting

Total RNA was isolated using Trizol (Invitrogen, United States) and reverse-transcribed into cDNA using the First Strand cDNA Synthesis Kit (TransGen, China) following the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was routinely performed using the SYBR Premix Ex Taq qPCR kit (TaKaRa, Japan). The alanine aminotransferase (ALT), aspartate aminotransferase (AST), total triglycerides (TG), total cholesterol (CHOL), superoxide dismutase (SOD), malondialdehyde (MDA), and H_2O_2 concentrations were detected using test kits according to the manufacturer's instructions. Total protein was isolated using radioimmune precipitation assay buffer (TaKaRa, Japan) supplemented with a protease inhibitor (Roche, Switzerland). After quantification using the BCA Protein Assay Kit (Thermo, United States), the proteins were separated by sodium-dodecyl sulfate gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were blocked and incubated overnight with antibodies against PTEN (9188T, CST), p-AMPK (ab32047, Abcam), p-mammalian target of rapamycin (mTOR) (CSB-PA271384, Cusabio), LC3 (12741T, CST), p62 (ab91526, Abcam), and GAPDH (ab245355, Abcam) at 4 °C. The membranes were incubated with HRP-conjugated secondary antibodies (ab205718, Abcam). Finally, the protein bands were detected using enhanced chemiluminescence (ECL) kits (Thermo, United States).

Hematoxylin and eosin staining and oil red staining

The hematoxylin and eosin (HE) staining was performed using the HE staining kit (C0105S, Beyotime) according to the manufacturer's instructions. Oil red staining was performed using an oil red staining solution (G 1262, Solarbio; C0157S, Beyotime). Briefly, 5-10 μm thick fresh frozen tissue was placed on the slide and dried at room temperature for 30-60 min. The sections were fixed with 10% paraformaldehyde for 10 min, washed thrice with distilled water, and dried for several minutes. After that, the oil red was diluted with deionized water in a 3:2 ratio, with impurities removed by filter paper, and left for 10 min at room temperature. Preheated oil red was used for tissue dye in a 6 °C temperature box for 8-10 min. After the 85% propylene glycol solution was differentiated for 2-5 min, it was washed twice with distilled water and restained with hematoxylin for 30 s. After rinsing with running water for 3 min, the tablets can be sealed with glycerine gelatin.

Luciferase reporter assay and TEM

The luciferase reporter assay was performed according to the manufacturer's instructions of Pierce™ Cypridina-Firefly Luciferase Dual Assay Kit (16184, Thermo). Briefly, AML-12 cells were co-transfected with a 10 nM miR-103 or NC control, a 2 ng pRL-CMV, and a 20 ng firefly luciferase reporter plasmid containing PTEN of the wild-type or mutant 30-untranslated region. Then, 48 h after transfection, the cell lysates were determined by luciferase to observe the interaction between miR-103 and PTEN. The liver tissues with the size of 1 mm × 1 mm × 1 mm were fixed, dehydrated, impregnated, and embedded to make ultrathin sections (50-70 nm) and then stained with uranium acetate and lead citrate successively and dried for observation under TEM.

Immunofluorescence

The frozen slices of 5-10 μm thick liver tissue were dried at room temperature for 30-60 min. They were sequentially fixed with 10% paraformaldehyde for 10 min, rinsed thrice with distilled water, and dried for several minutes. After that, the antigen was repaired by microwave at 92 °C-96 °C for 10-15 min, cooled to room temperature naturally, and sealed with 5% BSA at 37 °C for 60 min. After pouring the excess serum, LC3-II/I antibody was diluted at 1:100, added into samples, and incubated at 4 °C overnight. Rinsed with phosphate buffered saline (PBS) the next day, samples were added to the mixture of the fluorescent secondary antibody and 4',6-diamidino-2-phenylindole at a ratio of 1:200 and incubated for 60 min at room temperature. After washing with PBS, laser confocal scanning for immunofluorescence was performed.

Transwell analysis

3T3-L1 cells were first routinely induced to differentiate into adipocytes to identify the transfer of miR-103 from adipocytes to AML-12 cells. After that, Cy3-labeled miR-103 was transfected and then co-cultured with the underlying AML-12 cells through a transwell chamber. AML-12 cells were then isolated, and immunofluorescence determined the red fluorescence value.

Cell transfection

Cells for transfection were incubated into a 6-well plate. 5-10 μL miR-NC-anta and miR-103-anta were absorbed and diluted into 250 μL Opti-MEMI reduced serum medium, mixed gently, and incubated at room temperature for 5 min. Then, 3-6 μL Lipofectamine® 2000 Reagent was diluted to 250 μL of Opti-MempI reduced-serum medium, lightly mixed, and incubated at room temperature for 5 min. Diluted miR-NC-anta, miR-103-anta, and diluted Lipofectamine® 2000 Reagent were carefully mixed, gently blended, and incubated at room temperature for 20 min to form the reagent complex. After that, cells were washed with 2 mL serum-free medium, added with 2 mL of Opti-MEMI low serum medium to each well, and then added to 500 μL of miR-NC-anta and miR-103-anta-Lipofectamine® 2000 Reagent complex. These reagents were gently mixed and prepared for use.

Statistical analysis

All data are presented as mean ± SD. Differences between the two groups were analyzed using the student's *t*-test for categorical data and the chi-square method for numerical data. All statistical analyses were performed using GraphPad 9.0.2 software. Statistical significance between groups was set at $P < 0.05$.

RESULTS

The expression of miR-103 was increased in NASH mice, and inhibition of miR-103 could alleviate NASH

First, we successfully constructed animal models of NASH. Compared with the control, ALT, AST, TG, and CHOL were increased in NASH mice (Figure 1A). Hepatocyte ballooning, inflammatory cell infiltration, and hepatic lipid accumulation were observed in the livers of NASH mice (Figures 1B and C). Furthermore, we successfully extracted and confirmed AT-derived and circulating exosomes (Supplementary Figure 1). Subsequently, the expression of miR-103 in the livers, AT-derived exosomes, and circulating exosomes was detected by qRT-PCR. MiR-103 expression levels in the livers, AT-derived exosomes, and circulating exosomes were significantly increased in the NASH model group compared with the control group. Antagonizing miR-103 decreased miR-103 expression in NASH mice, but miR-NC-anta had no significant effect in NASH mice (Figure 1D). In addition, compared with miR-NC-anta and the model group, miR-103-anta treatment significantly reduced serum ALT and AST, decreased serum CHOL and TG, and alleviated oxidative stress (Figure 1A). Histologically, HE and oil red staining of the liver also indicated that inhibition of miR-103 alleviated hepatocyte ballooning, inflammatory cell infiltration, and hepatic lipid accumulation. (Figures 1B and C). Our above results indicate that the miR-103 level is elevated in NASH model mice, and reducing the expression of miR-103 can alleviate NASH, suggesting the potential involvement of miR-103.

The interaction between miR-103 and PTEN is involved in the process of autophagy

To further investigate the regulatory mechanism of miR-103 on NASH, we used *TargetScan* to predict its downstream targets[16]. The predicted results showed that PTEN, a gene that plays an essential role in autophagy, might interact with miR-103. Therefore, we used a dual-luciferase reporting experiment to further confirm their interaction (Figure 2A). The wild-type PTEN could interact with miR-103, while the mutant PTEN could not. Next, the protein content of PTEN and autophagy-related proteins such as p-AMPKa, p-mTOR, LC3-II/I, and p62 were determined by western blot. The results showed that compared with the control group, the expressions of p-AMPKa, p-mTOR, and p62 were significantly

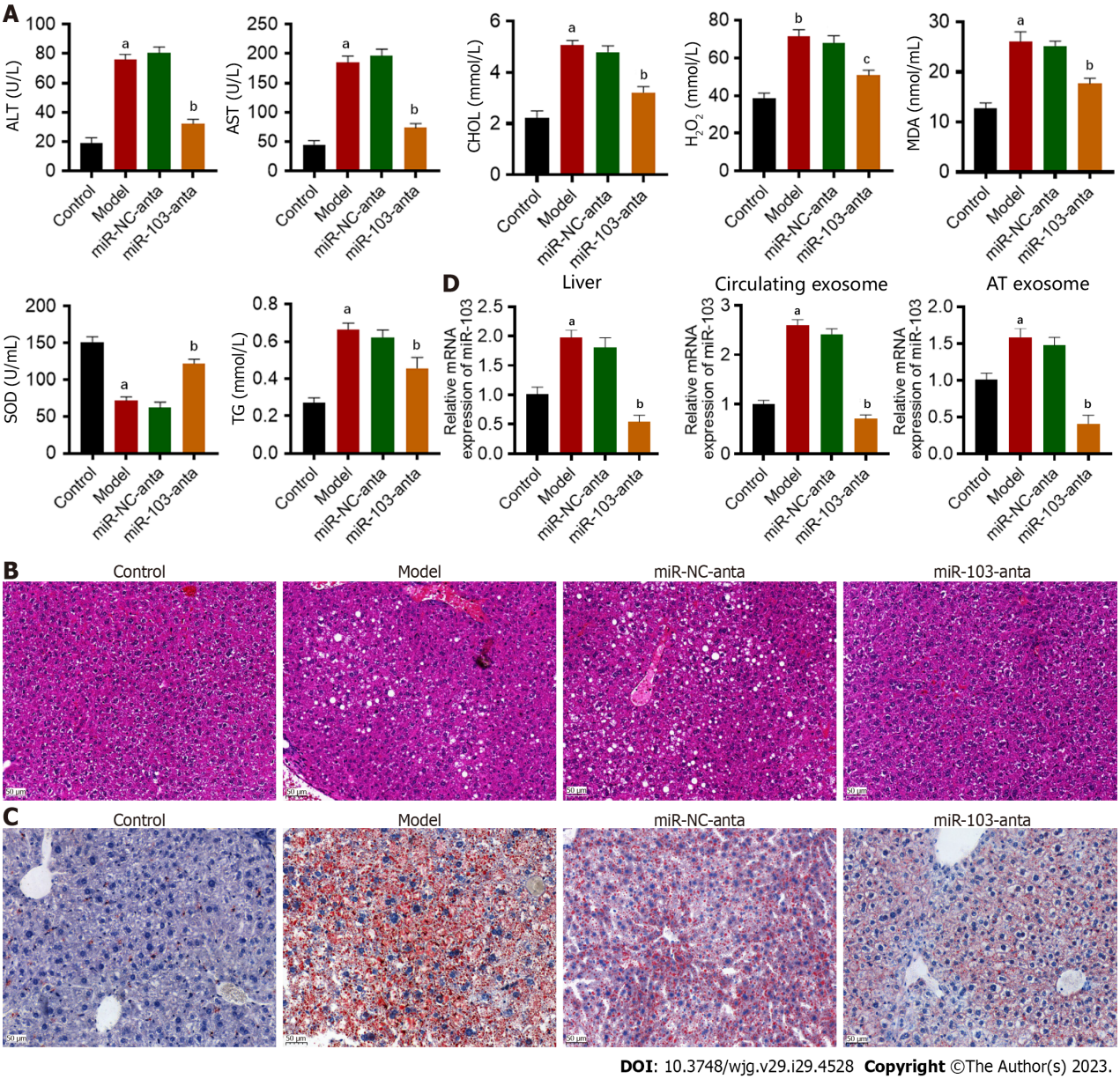

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Figure 1 The effect of miR-103 on the development of non-alcoholic steatohepatitis in mice. A: The results of ELISA to measure serum alanine aminotransferase (ALT), aspartate aminotransferase, total triglycerides, total cholesterol, superoxide dismutase, malondialdehyde, and H_2O_2; B: Hematoxylin and eosin staining of mice liver from different groups; C: Oil-red staining of mice liver from different groups; D: The expression of miR-103 in the liver, circulating exosomes, and adipose tissue-derived exosomes. $^{a}P < 0.01$ *vs* control; $^{b}P < 0.01$ *vs* model. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Total triglycerides; CHOL: Cholesterol; SOD: Superoxide dismutase; MDA: Malondialdehyde.

increased in the model group, while the protein levels of PTEN and LC3-II/I were decreased (Figure 2B). In addition, TEM and immunofluorescence staining showed that the number of autophagosomes in the liver of NASH model mice was significantly reduced compared with control mice (Figures 2C and D). However, compared with the model and miR-NC-anta groups, p-AMPKa, p-mTOR, p62, and the number of autophagosomes were significantly decreased in the miR-103-anta group. Similarly, the protein levels of PTEN and LC3-II/I was increased (Figures 2C and D). All these results indicate that autophagy is inhibited in the development of NASH, while miR-103-anta treatment could antagonize those changes. These results indicate that miR-103 interacts with PTEN and interferes with the downstream autophagy process, suggesting that the inhibition of autophagy in NASH may be attributed to the increased expression of miR-103.

MiR-103 participates in the development of NASH by inhibiting autophagy via interacting with PTEN

We then conducted *in vitro* experiments to further confirm our hypothesis. The results showed that inhibition of miR-103 expression could reduce the accumulation of lipids in NASH model cells, decrease the release of ALT and AST, and relieve oxidative stress. However, the effect of miR-103-anta was partially eliminated by silencing PTEN (Figures 3A and B). The above results indicate that miR-103 is involved in NASH formation partially through its interaction with PTEN. In addition, we detected the expression of autophagy-related proteins. We found that the expression of p-AMPKa, p-mTOR, and p62 was increased in NASH cells, while the expression of PTEN and LC3-II/I was decreased. Treatment with miR-

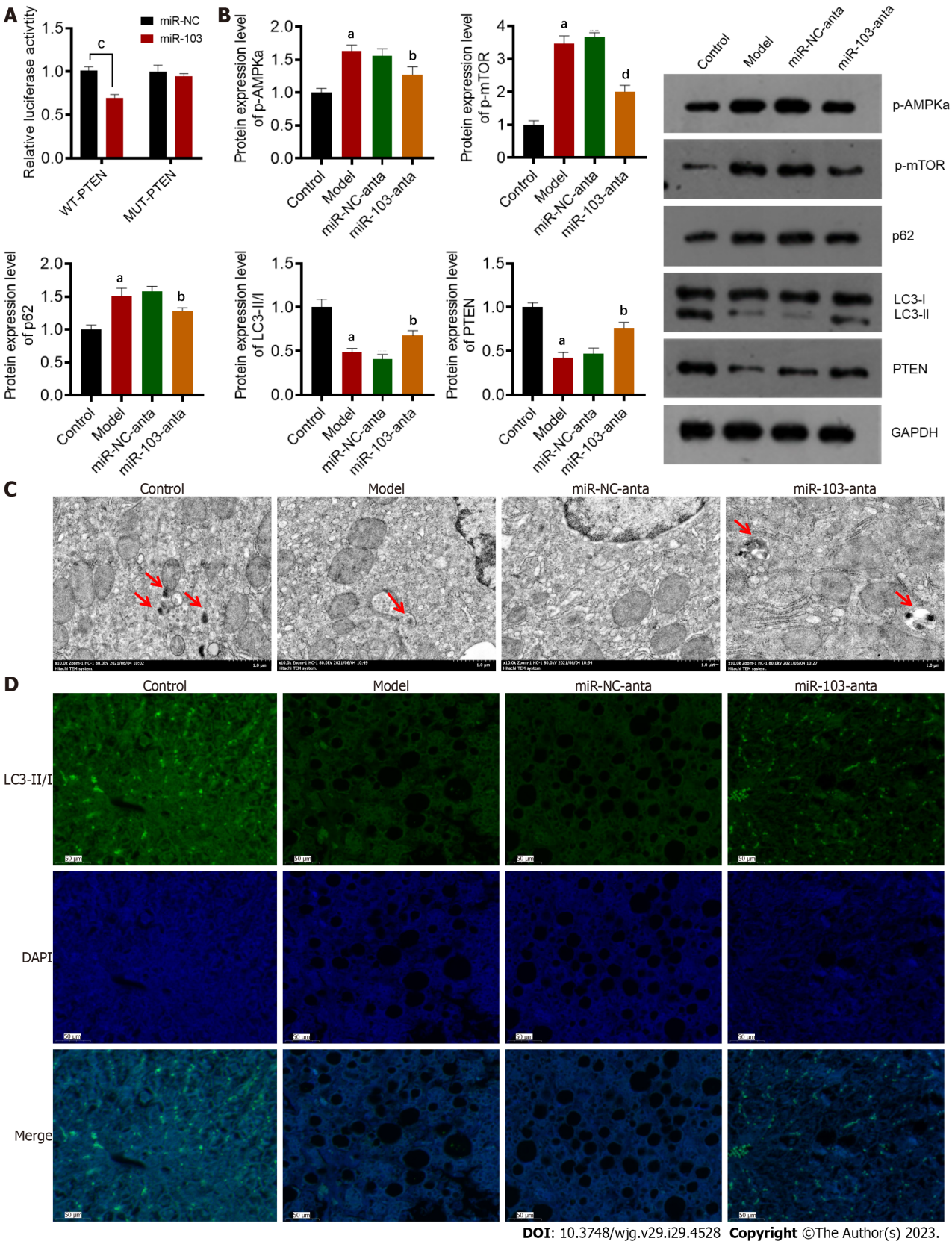

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Figure 2 The capacity of miR-103 in targeting phosphatase and tensin homolog gene and affecting autophagy. A: The luciferase reporter assay results verified the interaction between miR-103 and phosphatase and tensin homolog; B: Western blotting showed the differential expression of the autophagy-related protein; C: Transmission electron microscopy images of autophagosomes (red arrowhead) in the liver; D: The results of immunofluorescence staining showed liver autophagosomes in different groups. $^{a}P < 0.01$ *vs* control; $^{b}P < 0.01$ *vs* model; $^{c}P < 0.01$ *vs* miR-103; $^{d}P < 0.05$ *vs* model. PTEN: Phosphatase and tensin homolog; WT: Wild type; MUT: Mutant; DAPI: 4',6-diamidino-2-phenylindole; mTOR: Mammalian target of rapamycin; NC: Negative control.

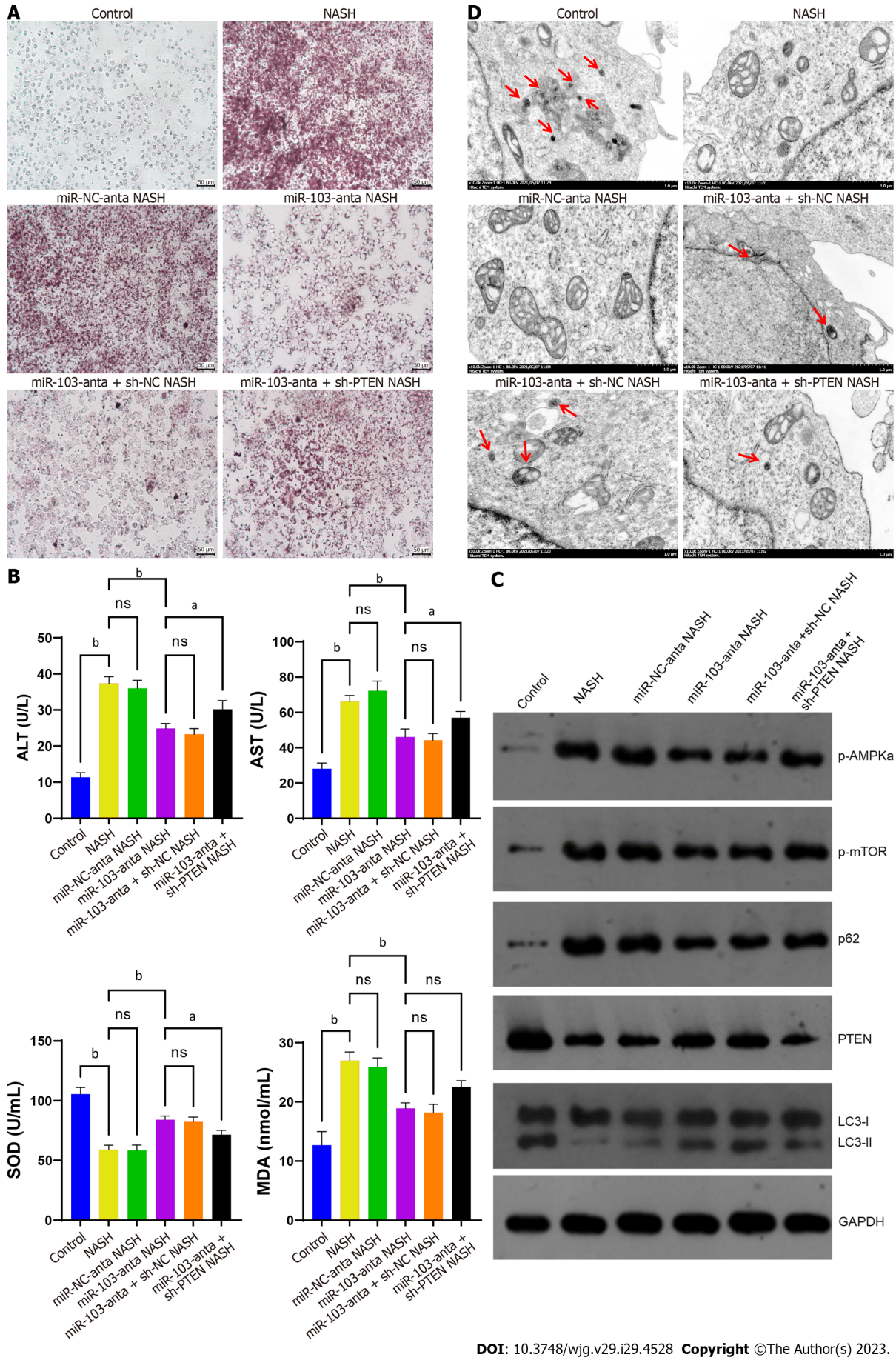

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Figure 3 *In vitro* experiments confirmed the interaction between miR-103 and phosphatase and tensin homolog gene. A: Oil-red staining of

cells from different groups showed different lipid droplet accumulation and inflammatory cell infiltration; B: The results of ELISA to measure serum alanine aminotransferase, aspartate aminotransferase, superoxide dismutase and malondialdehyde; C: Western blotting detected the expression of the autophagy-related protein of cells from different groups; D: Transmission electron microscopy pictures of autophagosomes (red arrowhead) in cells from different groups. $^{a}P < 0.05$, $^{b}P < 0.01$, ns: No significance. NASH: Non-alcoholic steatohepatitis; PTEN: Phosphatase and tensin homolog; NC: Negative control; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Total triglycerides; CHOL: Cholesterol; SOD: Superoxide dismutase; MDA: Malondialdehyde.

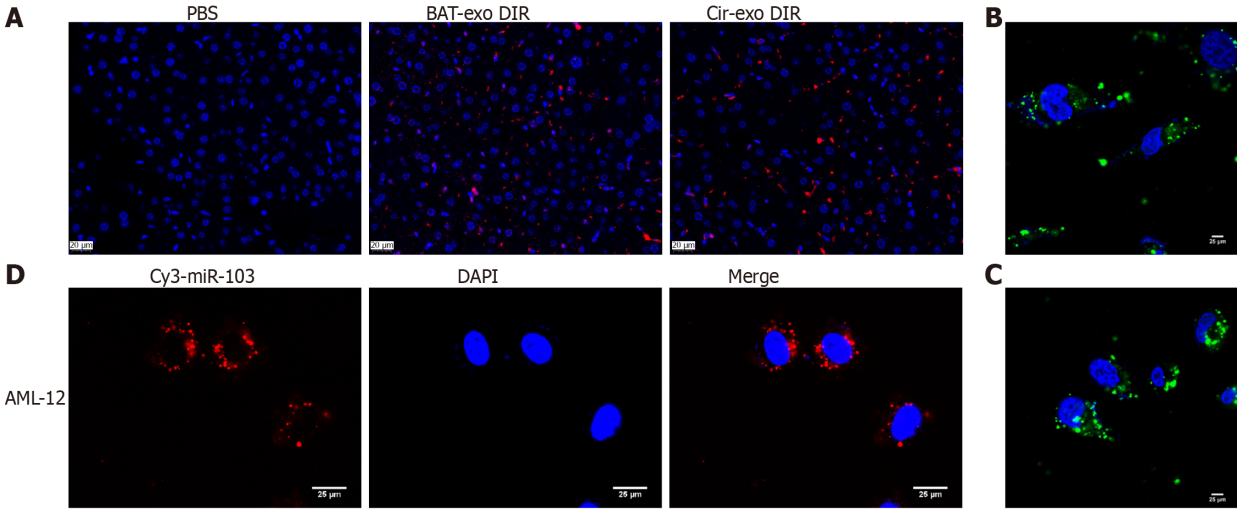

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Figure 4 MiR-103 transfers from adipocytes to hepatocytes. A: The fluorescence of liver tissue of mice injected with adipose tissue (AT)-exosomes or phosphate buffered saline; B: AT exosomes can be absorbed into alpha mouse liver 12 (AML-12) cells; C: Circulating exosomes can be absorbed into AML-12 cells; D: Transewell assay confirmed that miR-103 could be transferred from adipocytes to AML-12 cells. BAT: Brown adipose tissue; Exo: Exosomes; DIR: 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyaine iodide; Cir: Circulating; PBS: Phosphate buffered saline; DAPI: 4',6-diamidino-2-phenylindole; AML-12: Alpha mouse liver 12.

103-anta elevated the expression of PTEN and LC3-II/I and reduced the expression of p-AMPKa, p-mTOR, and p62 in NASH cells, while PTEN silencing inhibited the effect of miR-103-anta (Figure 3C). Finally, we also found that the number of autophagosomes decreased in NASH cells, and such declination was partially antagonized after the inhibition of miR-103. Similarly, PTEN silencing inhibited the effect of miR-103-anta (Figure 3D). To sum up, we confirmed the role of the miR-103-PTEN-autophagy axis in NASH through *in vitro* experiments.

Hepatocyte absorption of AT-derived exosome miR-103

To confirm that AT-derived exosomes can encapsulate miR-103 and target the liver, we conducted *in vivo* and *in vitro* experiments. In the *in vivo* experiment, AT and circulating exosomes of mice were extracted. The exo-DIR complex collected by centrifugation was injected into the tail vein of mice, and the PBS group was used as a control. The fluorescence of liver tissue was observed after mice scarification. There was no fluorescence in the PBS group but observed in AT-exo-DIR and cir-exo-DIR groups, with similar fluorescence intensity (Figure 4A). In the part of the *in vitro* experiment, the AT exosomes and circulating exosomes were detected by the FISH probe to migrate into AML-12 cells (Figures 4B and C). Further transewell assay showed that Cy3-labeled miR-103 could be transferred from adipocytes to AML-12 cells (Figure 4D).

Role of AT-derived exosome miR-103 in NASH

To verify the role of AT-derived exosome miR-103 in NASH through *in vivo* experiments, we extracted exosomes from mouse AT. We injected the extracted exosomes without other treatments and with miR-103-anta or miR-NC-anta into mice through the tail vein. Firstly, we found that injection of AT-derived exosomes increased the level of miR-103 in the liver, serum, and AT compared with the control group (Figure 5A). In addition, compared with the control group, treating AT-derived exosomes can increase serum ALT, AST, CHOL, and TG levels and aggravate oxidative stress (Figure 5B). Histologically, HE and oil red staining of the liver showed that treating AT-derived exosomes could aggravate hepatocyte ballooning, inflammatory cell infiltration, and intrahepatic lipid accumulation (Figures 5C and D). However, miR-103-anta treatment could partially eliminate such an effect while miR-NC-anta treatment could not, suggesting that AT-derived exosome aggravates NASH, and this effect is partly dependent on its encapsulated miR-103.

AT derived-exosome miR-103 inhibits autophagy in hepatocytes

To further verify *in vivo* that miR-103 in AT-derived exosomes is involved in NASH formation by influencing autophagy, we detected the expression of autophagy-related proteins in the liver of mice in each group. We found that compared with the control group, the expression of p-AMPKa, p-mTOR, and p62 in the livers of AT exosome-treated group was increased, while the expression of PTEN and LC3-II/I was decreased. Further miR-103-anta treatment increased the

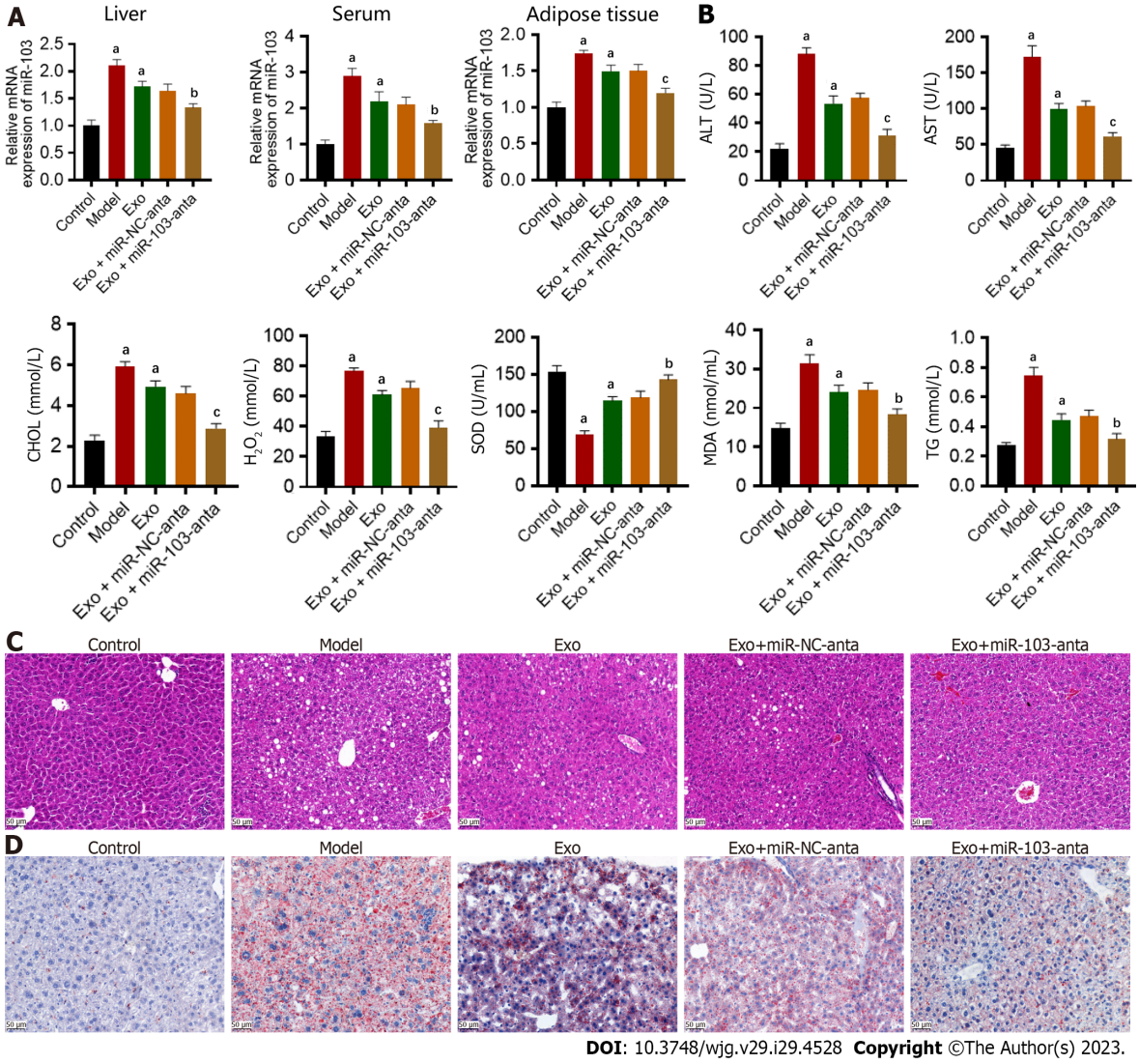

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Figure 5 The effect of adipose tissue-derived exosomes miR-103 on the development of non-alcoholic steatohepatitis in mice. A: The expression of miR-103 in the liver, serum, and adipose tissue; B: The results of ELISA to measure serum alanine aminotransferase, aspartate aminotransferase, total triglycerides, total cholesterol, superoxide dismutase, malondialdehyde, and H_2O_2; C: Hematoxylin and eosin staining of mice liver from different groups; D: Oil-red staining of mice liver from different groups. $^{a}P < 0.01$ *vs* control; $^{b}P < 0.05$ *vs* exosomes; $^{c}P < 0.01$ *vs* exosomes. Exo: Exosomes; NC: Negative control; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Total triglycerides; CHOL: Cholesterol; SOD: Superoxide dismutase; MDA: Malondialdehyde.

expression level of PTEN and LC3-II/I and reduced the expression level of p-AMPKa, p-mTOR, and p62, while miR-NC-anta had no similar effect (Figures 6A and B). Finally, through TEM and immunofluorescence, we found that the number of autophagosomes in the livers of mice treated with AT-derived exosomes was decreased, while miR-103-anta could partially antagonize such effect (Figures 6C and D).

DISCUSSION

NASH is considered the watershed in the progress of NAFLD, which is more closely related to the occurrence of liver cirrhosis and other complications. The prevalence of NASH has been increasing in recent years, resulting in a big challenge in disease burden and patient suffering. For instance, the lifetime cost of care for patients with NASH was around US $222 billion in 2017 in the United States[17]. More intriguingly, many NASH patients are obese and tend to have higher healthcare costs than non-obese NASH patients[18], but the etiology is still vague. Therefore, it is necessary to study the role and underlying mechanisms of obesity on NASH and further provide possible therapeutic targets. In this study, we confirmed the important role of AT-derived exosomes miR-103 in NASH and preliminary revealed its regulation on hepatocyte autophagy through targeting PTEN, which might partially provide the mechanisms by which obesity affects NASH.

MiRNA belongs to the family of non-coding RNA, which generally consists of 22 nucleotides[19] and regulates the mRNA levels of target genes[20]. Previous studies have identified a variety of miRNAs involved in metabolism-related diseases[21]. For example, miR-200 and miR-29 families play an important role in maintaining the balance between the

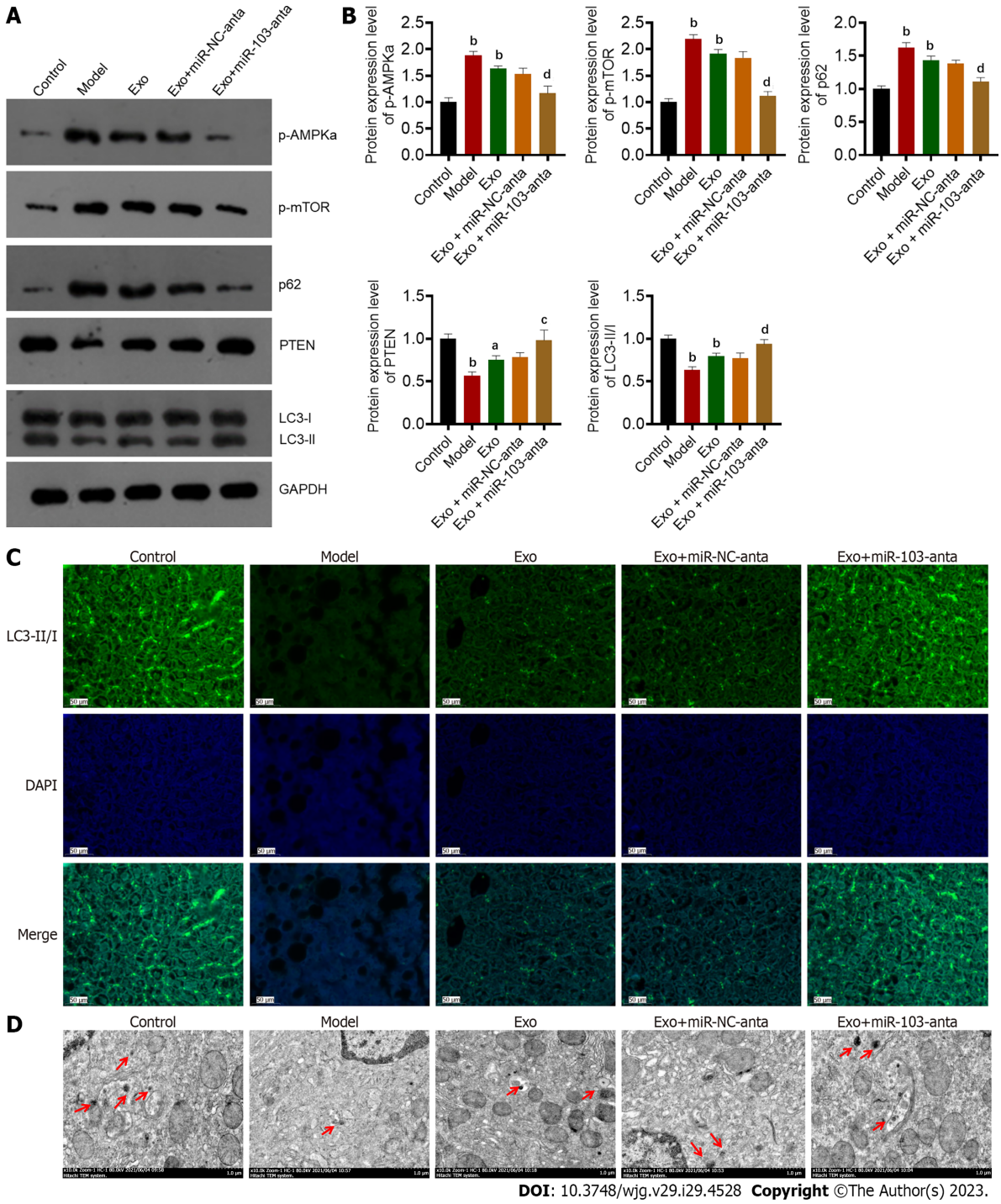

Figure 6 The effect of adipose tissue-derived exosomes miR-103 on autophagy in mice. A and B: Western blotting detected the expression of the autophagy-related protein; C: The results of immunofluorescence staining to observe autophagosomes in the liver from different groups; D: Transmission electron microscopy images of autophagosomes (red arrowhead) in the liver from different groups. $^{a}P < 0.05$ *vs* control; $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$ *vs* exosomes; $^{d}P < 0.01$ *vs* exosomes. Exo: Exosomes; PTEN: Phosphatase and tensin homolog; DAPI: 4',6-diamidino-2-phenylindole; mTOR: Mammalian target of rapamycin; NC: Negative control.

proliferation and differentiation of pancreatic β cells[22,23]. MiR-33a and miR-33b are involved in cholesterol and lipid metabolism[24,25]. Furthermore, several miRNAs are also targeting the liver to regulate metabolic processes. For instance, miR-122, one of the most abundant miRNAs in the liver, is involved in hepatic cholesterol and lipid metabolism [26,27]. Besides, miR-103, the focus of our study, has also been confirmed to be closely related to hepatic insulin sensitivity and the regulation of glucose homeostasis in previous studies[12]. Since NAFLD is the hepatic manifestation of metabolic syndrome, it is theoretically possible that miR-103 participates in NAFLD, but related research is still lacking. NASH was linked to menopause[28], and miR-103 was found to be linked to G protein-coupled estrogen receptor 1[29]. Therefore, the estrogen signaling pathway is the potential mechanism where miR-103 promotes NASH. However, in this study, for the first time, we showed that the miR-103 level in the liver of NASH mice was significantly increased while inhibiting miR-103 expression could alleviate NASH, suggesting that miR-103 is one of the potential targets for NASH

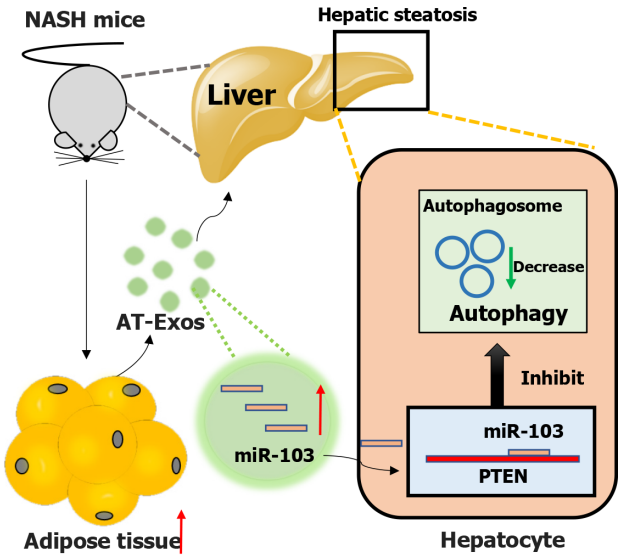

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Figure 7 The hypothesis of obesity-induced non-alcoholic steatohepatitis. The increased adipocyte tissue will secret more adipose tissue (AT)-derived exosomes, which include various contents, and miR-103 is our focus. The increased AT-derived exosomes miR-103 could be absorbed by hepatocytes and further regulate non-alcoholic steatohepatitis progression by affecting the autophagy process by targeting phosphatase and tensin homolog gene. AT: Adipose tissue; NASH: Non-alcoholic steatohepatitis; Exo: Exosomes; PTEN: Phosphatase and tensin homolog.

treatment.

We further identified the AT-derived exosomes as the source of miR-103 upregulation in the NASH mouse model. There have been many studies on the role of AT-derived exosomes in the development of NAFLD in obese people[30]. For instance, Fuchs *et al*[31] showed that the concentration of free exosomes is significantly higher in obese with NAFLD (OB-NAFLD) patients compared with lean with normal intrahepatic triglyceride content (LEAN) and obese with normal intrahepatic triglyceride content (OB-NL) populations and that these exosomes are at least partially derived from AT. Compared with exosomes derived from the LEAN and OB-NL groups, plasma and AT-derived exosomes from the OB-NAFLD group caused insulin resistance in both myotubes and hepatocytes, demonstrated by impaired insulin signaling. However, the underlying mechanism of the above effects of AT-derived exosomes in NAFLD patients has not been further explored. Our results complement this by revealing the effect of AT-derived exosomes through the miR-103-PTEN pathway, which broadens our understanding of NASH pathogenesis from the angle of trans-cellular crosstalk.

We also confirmed that autophagy is the downstream of the action of miR-103 through *in vivo* and *in vitro* experiments. Autophagy is an evolutionarily conserved cellular degradation process that delivers some intracellular components to lysosomes for degradation[32]. Current studies suggest that autophagy includes three subtypes: Macroautophagy, microautophagy, and chaperon-mediated autophagy[33]. Autophagy plays a vital role in the liver. It involves many basic liver functions, such as glycogenolysis, gluconeogenesis, and β-oxidation[34]. Previous studies have also shown that autophagy is hampered in NAFLD patients. Our previous study also revealed that autophagy inhibition plays an important role in NASH development[35]. Furthermore, restoring autophagy through certain drugs (trehalose, rapamycin, carbamazepine, or other pharmaceutical agents) or gene targets (overexpression of Atg7 or TFEB) can also alleviate NAFLD[36]. In addition, thyroxine[37] and caffeine[38] were also identified to reduce NAFLD by regulating liver autophagy. Therefore, miR-103 is expected to be one of the therapeutic targets for its autophagy regulation capacity and needs further clinical investigation in the future.

Some limitations in this study should be acknowledged. Firstly, AT-derived exosomes contain many non-coding RNAs, and we did not detect changes in the expression of other non-coding RNAs. Moreover, inhibition of miR-103 only partially inhibited the effect of AT-derived exosomes. Therefore, the above results suggest that AT-derived exosomes promote the development of NASH in multiple ways, and miR-103 is only one of them. Further research is needed on other reasons why AT-derived exosomes promote the development of NASH. Secondly, we did not design *in vivo* experiments to verify whether inhibition of autophagy could abolish the role of miR-103 in NASH. Therefore, in future studies, we may improve this part of the experiment and further explore the mechanism of miR-103 affecting autophagy. Finally, the preliminary data on the hepatocyte absorption of AT-derived exosome miR-103 needs further verification.

CONCLUSION

To sum up, our study confirms the important role of miR-103-PTEN - autophagy axis in NASH, and the elevation of miR-103 in the liver of the NASH model is partly due to hepatocyte absorption of AT derived-exosomes, which also partially explains the underlining mechanism of obesity leading to NASH (Figure 7).

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic steatohepatitis (NASH) has become one of the leading causes of cirrhosis and the second leading cause of liver transplantation. miR-103 is involved in regulating insulin sensitivity, a common manifestation of metabolic syndrome in patients with NASH.

Research motivation

The specific role of miR-103 in the development of NASH also deserves further study.

Research objectives

To explore the specific role of miR-103 in the development of NASH and provide new therapeutic targets for NASH.

Research methods

The expression levels of miR-103 were detected and compared between NASH mice and control. The effect of miR-103 on NASH progression was explored by miR-103 antagonizing, including both changes of steatosis and inflammation degree. The interaction between miR-103 and the autophagy-related gene phosphatase and tensin homolog (PTEN) was confirmed by dual-luciferase reporter assay. The role of the interaction between miR-103 and PTEN on autophagy was verified in NASH cells. Finally, the effects of miR-103 from adipose tissue (AT)-derived exosomes on NASH and autophagy were analyzed through animal experiments.

Research results

The expression of miR-103 was increased in NASH mice, compared with the control, and inhibition of miR-103 could alleviate NASH. MiR-103 could interact with PTEN. MiR-103-anta inhibited autophagy in NASH mice. Further experiments showed PTEN silencing inhibited the effect of miR-103-anta. AT-derived exosome miR-103 aggravated NASH and inhibited autophagy in mice.

Research conclusions

AT derived-exosome increased the levels of miR-103 in the liver, and miR-103 aggravated NASH. Mechanically speaking, miR-103 could interact with PTEN and inhibit autophagy.

Research perspectives

MiR-103 may be a potential target for NASH treatment in the future.

FOOTNOTES

Author contributions: Jin X designed the report; Lu MM performed experiments, wrote the paper and assisted to design the report; Ren Y and Zhou YW performed experiments and statistical analysis; Zhang MM, Ding LP, and Cheng WX assisted to perform experiments and analyzed data; and all authors had access to the study data and reviewed and approved the final manuscript.

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Institutional animal care and use committee statement: This study was carried out following the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The animal protocol was approved by the institutional review board of the Tab of Animal Experimental Ethical Inspection of the First Affiliated Hospital of Zhejiang University. The Reference Number is 2020-1407.

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

Data sharing statement: All the data included in this study are available upon request by contact with the corresponding author.

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

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ORIGINAL ARTICLE

Basic Study

MiR-204-3p overexpression inhibits gastric carcinoma cell proliferation by inhibiting the MAPK pathway and RIP1/MLK1 necroptosis pathway to promote apoptosis

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Abstract

BACKGROUND

Gastric carcinoma (GC) is the third most frequent cause of cancer-related death, highlighting the pressing need for novel clinical treatment options. In this regard, microRNAs (miRNAs) have emerged as a promising therapeutic strategy. Studies have shown that miRNAs can regulate related signaling pathways, acting as tumor suppressors or tumor promoters.

AIM

To explore the effect of miR-204-3p on GC cells.

METHODS

We measured the expression levels of miR-204-3p in GC cells using quantitative real-time polymerase chain reaction, followed by the delivery of miR-204-3p overexpression and miR-204-3p knockdown vectors into GC cells. CCK-8 was used to detect the effect of miR-204-3p on the proliferation of GC cells, and the colony formation ability of GC cells was detected by the clonal formation assay.

The effects of miR-204-3p on GC cell cycle and apoptosis were detected by flow cytometry. The BABL/c nude mouse subcutaneous tumor model using MKN-45 cells was constructed to verify the effect of miR-204-3p on the tumorigenicity of GC cells. Furthermore, the study investigated the effects of miR-204-3p on various proteins related to the MAPK signaling pathway, necroptosis signaling pathway and apoptosis signaling pathway on GC cells using Western blot techniques.

RESULTS

Firstly, we found that the expression of miR-204-3p in GC was low. When treated with the lentivirus overexpression vector, miR-204-3p expression significantly increased, but the lentivirus knockout vector had no significant effect on miR-204-3p. *In vitro* experiments confirmed that miR-204-3p overexpression inhibited GC cell viability, promoted cell apoptosis, blocked the cell cycle, and inhibited colony formation ability. *In vivo* animal experiments confirmed that miR-204-3p overexpression inhibited subcutaneous tumorigenesis ability in BABL/c nude mice. Simultaneously, our results verified that miR-204-3p overexpression can inhibit GC cell proliferation by inhibiting protein expression levels of KRAS and p-ERK1/2 in the MAPK pathway, as well as inhibiting protein expression levels of p-RIP1 and p-MLK1 in the necroptosis pathway to promote the BCL-2/BAX/Caspase-3 apoptosis pathway.

CONCLUSION

MiR-204-3p overexpression inhibited GC cell proliferation by inhibiting the MAPK pathway and necroptosis pathway to promote apoptosis of GC cells. Thus, miR-204-3p may represent a new potential therapeutic target for GC.

Key Words: miR-204-3p; Gastric carcinoma; MAPK signaling pathway; Apoptosis; Necroptosis

Core Tip: Gastric carcinoma (GC) is a global health problem that seriously endangers human life; therefore, it is important to identify effective treatment targets. In this regard, microRNAs (miRNAs) have emerged as a promising therapeutic strategy. Studies have shown that miRNAs regulated signaling pathways, acting as tumor suppressors or tumor promoters. In this study, we first verified the inhibitory effect of miR-204-3p overexpression on GC cells through *in vitro* and *in vivo* experiments. Simultaneously, miR-204-3p overexpression induced GC cell apoptosis by inhibiting the MAPK pathway and the necroptosis pathway. Thus, miR-204-3p may represent a new potential therapeutic target for GC.

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INTRODUCTION

Gastric carcinoma (GC) is a gastrointestinal tumor and the third major cause of cancer-related death[1,2]. Early clinical symptoms are mild or asymptomatic, resulting in difficult diagnosis and a low patient survival rate[3,4]. Currently, the clinical therapy of GC primarily consists of radiotherapy, chemotherapy, and surgical excision, but the therapeutic effect is unsatisfactory[5,6]. Therefore, feasible targeted therapies are particularly important for GC patients. As research has progressed, molecular targets have been found to have a role in the occurrence and development of GC.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by binding to the 3'-untranslated region of target mRNA[7]. With further study in genetic engineering, it has been confirmed that miRNAs regulate different signaling pathways to take part in important cellular processes[8]. miRNAs can act as tumor promoters or inhibitors to target mRNA to regulate GC proliferation, metastasis, angiogenesis, and drug resistance[9-11]. Consequently, it is necessary to strengthen miRNA research for the diagnosis and clinical treatment of GC.

MiR-204-3p has demonstrated efficacy in treating various pathologies, including retinopathy, diabetes, and cancer[12-15]. Crucially, miR-204-3p is underexpressed in melanoma, thyroid carcinoma, glioma and bladder carcinoma, and is related to patient prognosis[16-19]. However, the mechanism of miR-204-3p in GC remains unclear.

Our team's previous research confirmed that the expression of miR-204-3p in GC tissue is low and is associated with poor prognosis in GC patients. In addition, we also verified that KRAS is its direct target[20]. However, the anti-GC effect of miR-204-3p still requires further research to support the possibility of miR-204-3p becoming a new target for the treatment of GC. In this study, we focused on determining the impact of miR-204-3p on GC cells phenotype and its anti-GC molecular mechanism, to provide theoretical support for the treatment of GC by miR-204-3p.

Table 1 Primer sequences

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')
KRAS	TGTGGACGAATATGATCCAACA	GCAAATACACAAAGAAAGCCCT
ERK1	ATGTCATCGGCATCCGAGAC	GGATCTGGTAGAGGAAGTAGCA
ERK2	TACACCAACCTCTCGTACATCG	ATGTCTGAAGCGCAGTAAGATT
GAPDH	CACCCACTCCTCCACCTTTGA	TCTCTCTTCCTCTTGTGCTCTCTTGC
miR-204-3p	CAAGTCGCTGGGAAGGCAA	CAGTGCAGGGTCCGAGGT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

MATERIALS AND METHODS

Cell culture and cell transfection

A normal human gastric epithelial cell line (GES-1) and three GC cell lines (HGC-27, AGS and MKN-45) were acquired from BNCC (Beijing, China). MKN-45 and HGC-27 cells were cultured in RPIM-1640 medium, AGS cells in DMEM/F-12 medium and GES-1 cells in DMEM medium. All culture media were purchased from Gibco (United States), and were supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum. Genechem (Shanghai, China) provided the green fluorescent protein-labeled miR-204-3p overexpression lentiviral vector (OE group), miR-204-3p knockdown lentiviral vector (KD group) and empty lentiviral vector (NC group), which were then transfected into HGC-27, MKN-45 and AGS cells using the tool virus user manual as a guide.

CCK-8 assay

Cell viability was evaluated using the CCK-8 assay. Specifically, lentivirus-transfected AGS and HGC-27 cells were seeded into 96-well plates (5000 cells/well) and cultured for 24 h, 48 h and 72 h, respectively. Following this step, each well was treated with 10 μL CCK-8 reagent (MedChemExpress, United States), and a microplate reader was used to measure the value at 450 nm following incubation for 2 h at 37°C.

Colony formation assay

Lentivirus-transfected AGS and HGC-27 cells were inoculated into 6-well plates (500 cells/well), and fixed with 4% paraformaldehyde for 30 min after 2 wk of continuous culture. Next, the fixing solution was washed off and the cells were stained using 0.5% crystal violet for 10 min. Finally, the cell clones were photographed and statistically analyzed based on clone sizes (diameter > 1 mm).

Flow cytometric analysis

Cell cycle was confirmed using the cell cycle kit (KeyGEN BioTECH, China). Lentivirus-transfected HGC-27 and AGS cells were collected at 1 × 10^6 cells/mL, and then fixed overnight at 37°C with 4% paraformaldehyde. On the second day, the fixing solution was washed off with PBS and 500 μL cell cycle detection working solution (Rnase A:PI = 1:9) was added. The distribution of various groups of cells in the cell cycle was detected after they had reacted for 30 min.

The Annexin V-APC/7-AAD double staining kit (KeyGEN BioTECH, China) was used to confirm apoptosis (early apoptosis and late apoptosis) in each group. Lentivirus-transfected AGS and HGC-27 cells were collected, and 500 μL Binding Buffer, 5 μL Annexin V-APC and 5 μL 7-AAD were added sequentially and gently mixed. Apoptosis was observed after the cells had reacted for 10 min.

Western blot

The expression of related proteins in lentivirus-transfected AGS and HGC-27 cells was detected. Firstly, total protein was extracted with RIPA (Epizyme Biotech, China) from GC cells and their content was confirmed using the BCA assay (Epizyme Biotech, China). Next, the proteins were isolated and transferred onto a polyvinylidene fluoride membrane, which was sealed with 5% skim milk powder, soaked in primary antibody and incubated overnight. On the second day, it was washed with TBST and soaked in HRP-linked secondary antibody (1:1000, 7074/7076, CST, United States) for 1 h. Finally, protein bands were visualized with ECL reagent (KeyGEN BioTECH, China), and the gray values of the protein bands were analyzed using Image J. GAPDH or β-tubulin was used as an internal control to standardize target proteins. p-ERK1/2 (1:5000, 4370), ERK1/2 (1:5000, 9102), RIP1 (1:1000, 3493), p-RIP1 (1:1000, 65746), MLK1 (1:1000, 5029) and p-MLK1 (1:1000, 91689) antibodies were purchased from CST. BAX (1:2000, ab32503), Caspase-3 (1:1000, ab13847), BCL-2 (1:5000, ab182858) and KRAS (1:1000, ab275876) antibodies were purchased from Abcam.

Quantitative real-time polymerase chain reaction

The related genes expressed in each group of GC cells were determined using quantitative real-time polymerase chain reaction (qRT-PCR). Briefly, total RNA was extracted from GC cells using TRIzol reagent (Invitrogen, United States), then cDNA was synthesized (Takara Bio, Japan) and gene expression levels were measured (Takara Bio, Japan). The primer sequences are shown in Table 1. U6 or GAPDH was used as a housekeeping gene, and target genes were calculated using

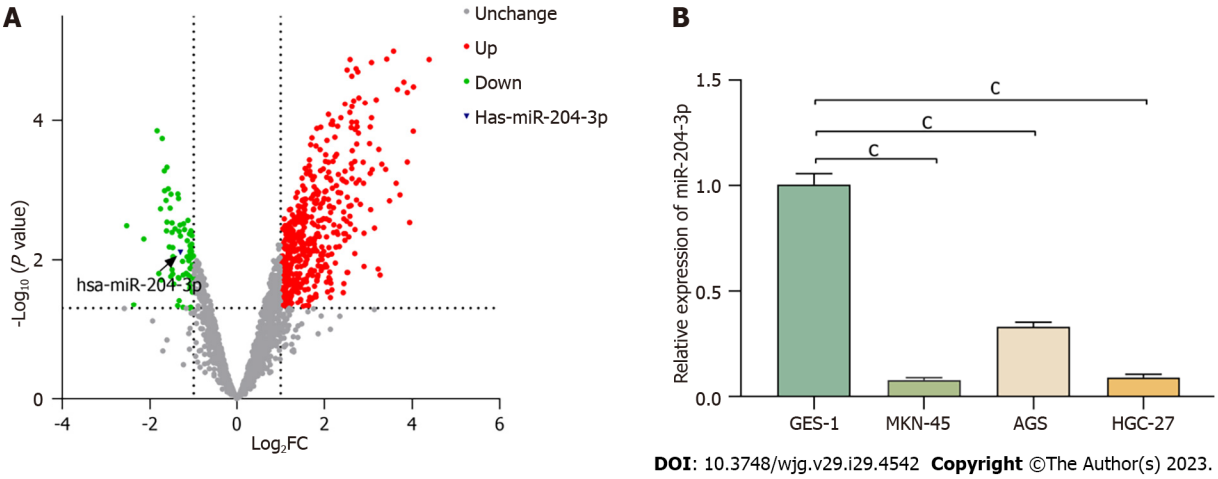

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Figure 1 Expression of miR-204-3p in gastric carcinoma. A: The expression level of miR-204-3p in gastric carcinoma tissues and para-carcinoma tissues was analyzed by the GEO database; B: The expression level of miR-204-3p in normal gastric mucosal epithelial cells and gastric carcinoma cells, $^{c}P < 0.001$.

the $2^{-\Delta\Delta Ct}$ method.

Tumor xenograft experiment

BABL/c nude mice (male, 4 wk, SPF) were provided by the Animal Laboratory Center of Ningxia Medical University. The animal protocol (IACUC-NYLAC-2022-251) was approved by the Institutional Animal Care and Use Committee of Ningxia Medical University. Following 7 d of adaptive feeding in BALB/c nude mice, lentivirus-transfected MKN-45 cells were cultured, and the cell concentration adjusted to 5×10^7 cells/mL. A suspension containing 100 μL cells was slowly subcutaneously injected into the back of nude mice, which were then returned to their cage for feeding. Growth of the subcutaneous tumor and mouse body weight were observed daily. The tumor volume (V) was calculated by $V = (W^2 \times L)/2$ (long diameter: L; short diameter: W). When the tumor on the back grew to an appropriate size and conformed to animal ethics, the animals were killed by CO_2 and photographed.

Differential expression analysis of miRNAs

We used the GEO database (https://www.ncbi.nlm.nih.gov/geo/) to screen differentially expressed miRNAs. We searched the keywords " miRNAs" and "gastric carcinoma, and downloaded Differential expression microarrays of miRNAs (series: GES79973). Then adjusted for $P < 0.05$, $|\log_2 (\text{fold change})| > 1$, and analyzed significant miRNAs.

Statistical analysis

The statistical methods used in this study were reviewed by Li-Qun Wang from the Department of Epidemiology, Department of Medical Statistics, Institute of Public Health and Management, Ningxia Medical University. All data represent the mean ± SD of at least three independent samples. Statistical analysis was conducted using SPSS 27.0 and GraphPad Prism 8.0. One-way analysis of variance was used to analyze the differences between groups. $P < 0.05$ indicated a statistically significant difference.

RESULTS

MiR-204-3p was underexpressed in GC

In our previous study, we analyzed 40 pairs of tissue samples and discovered that miR-204-3p expression was lower in GC tissues compared to normal tissues[20]. To further validate this finding, we utilized the GEO database to identify differentially expressed miRNAs in GC tissues and paracancerous tissues, which confirmed the downregulation of miR-204-3p in GC tissues (Figure 1A). Subsequently, we investigated miR-204-3p expression between GC cell lines (AGS, MKN-45 and HGC-27) and the normal gastric epithelial cell line GES-1. The results revealed that miR-204-3p was underexpressed in MKN-45, AGS, and HGC-27 cells compared to GES-1 cells (Figure 1B).

MiR-204-3p inhibited GC cell viability

Lentivirus transfection caused miR-204-3p overexpression and miR-204-3p knockdown in AGS and HGC-27 cells. The transfection rate was found to be approximately 90% after 72 h, indicating high transfection efficiency (Figure 2A). The qRT-PCR results demonstrated that the miR-204-3p overexpression lentivirus significantly upregulated its expression compared to the NC group in AGS and HGC-27 cells, but the miR-204-3p knockdown lentivirus did not result in downregulation of its expression (Figure 2B and C). These findings indicated that miR-204-3p overexpression in GC cells was highly satisfactory, while miR-204-3p knockdown does not yield meaningful outcomes. Subsequently, the cell viability assay showed that miR-204-3p overexpression significantly inhibited GC cell viability (Figure 2D and E).

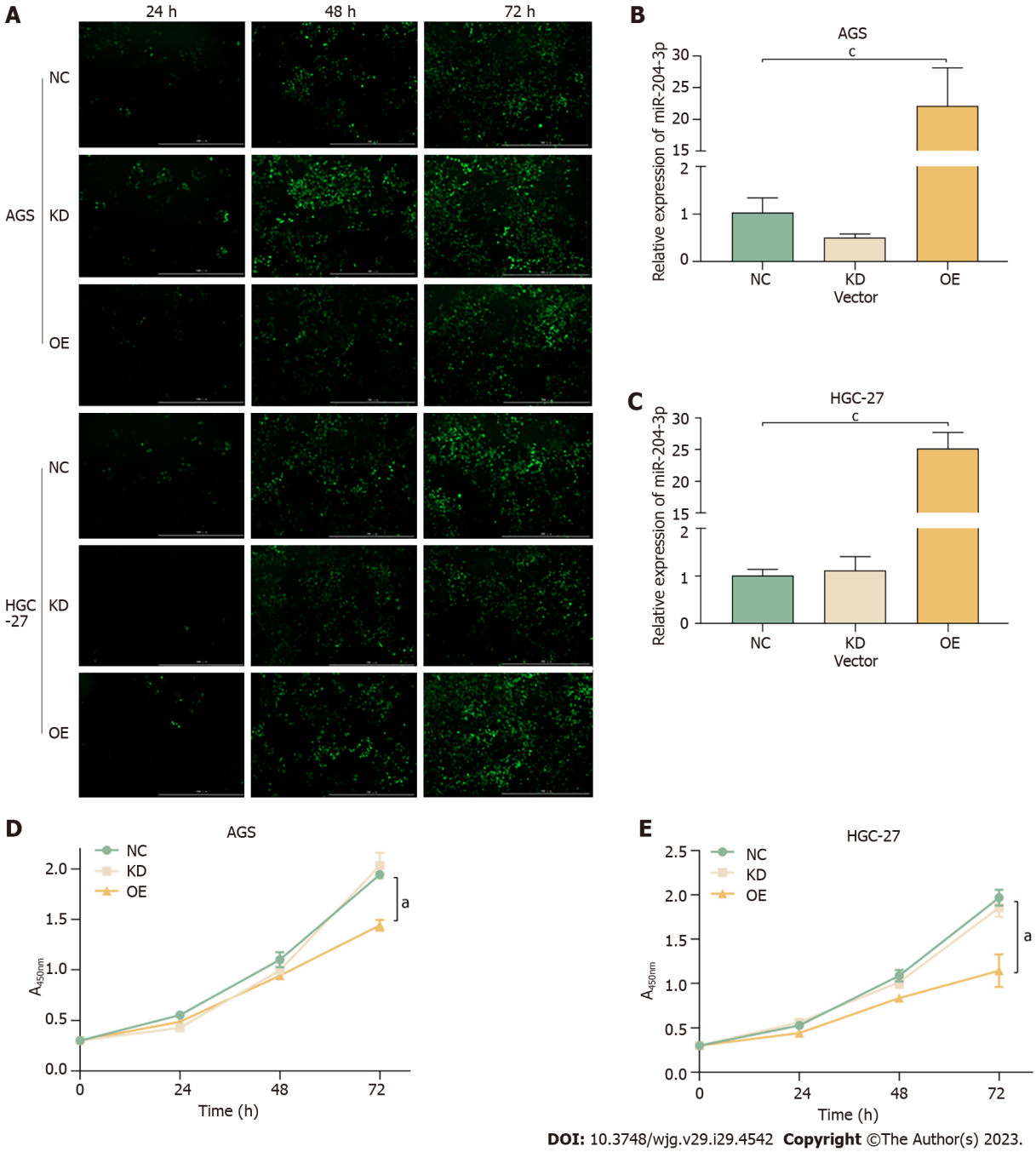

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Figure 2 Effect of miR-204-3p on AGS and HGC-27 cell viability. A: Transfection efficiency of lentiviral vector. Scale bar: 1000 μm, with representative images shown, fluorescence distribution of green fluorescent protein in cells indicating transfection efficiency; B and C: The expression level of miR-204-3p in lentivirus transfected AGS and HGC-27 cells; D and E: Effect of miR-204-3p on AGS and HGC-27 cell viability. $^{a}P < 0.05$, $^{c}P < 0.001$. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

MiR-204-3p inhibited the colony forming ability of GC cells

The impact of miR-204-3p on the colony forming ability of GC cells was evaluated. The crystal violet positive staining in the miR-204-3p overexpression group decreased (Figure 3A and B), and simultaneously, the number of clones formed in AGS and HGC-27 cells was distinctly reduced compared to the NC group (Figure 3C and D). This indicated that miR-204-3p overexpression inhibited the colony forming ability of GC cells.

MiR-204-3p promoted GC cell apoptosis

We evaluated apoptosis by collecting cells from each group (Figure 4). The apoptosis rates of AGS cells were 2.53 ± 0.12%, 3.73 ± 0.83% and 10.6 ± 0.70% in the NC, KD and OE groups, respectively (Figure 4A and C). Among HGC-27 cells, the apoptosis rates were 9.47 ± 0.58%, 7.87 ± 1.63% and 18.40 ± 1.27% in the NC, KD and OE groups, respectively (Figure 4B and D). The findings revealed that HGC-27 and AGS cells with miR-204-3p overexpression had a notably higher apoptosis rate, which indicated that miR-204-3p overexpression stimulated GC cell apoptosis.

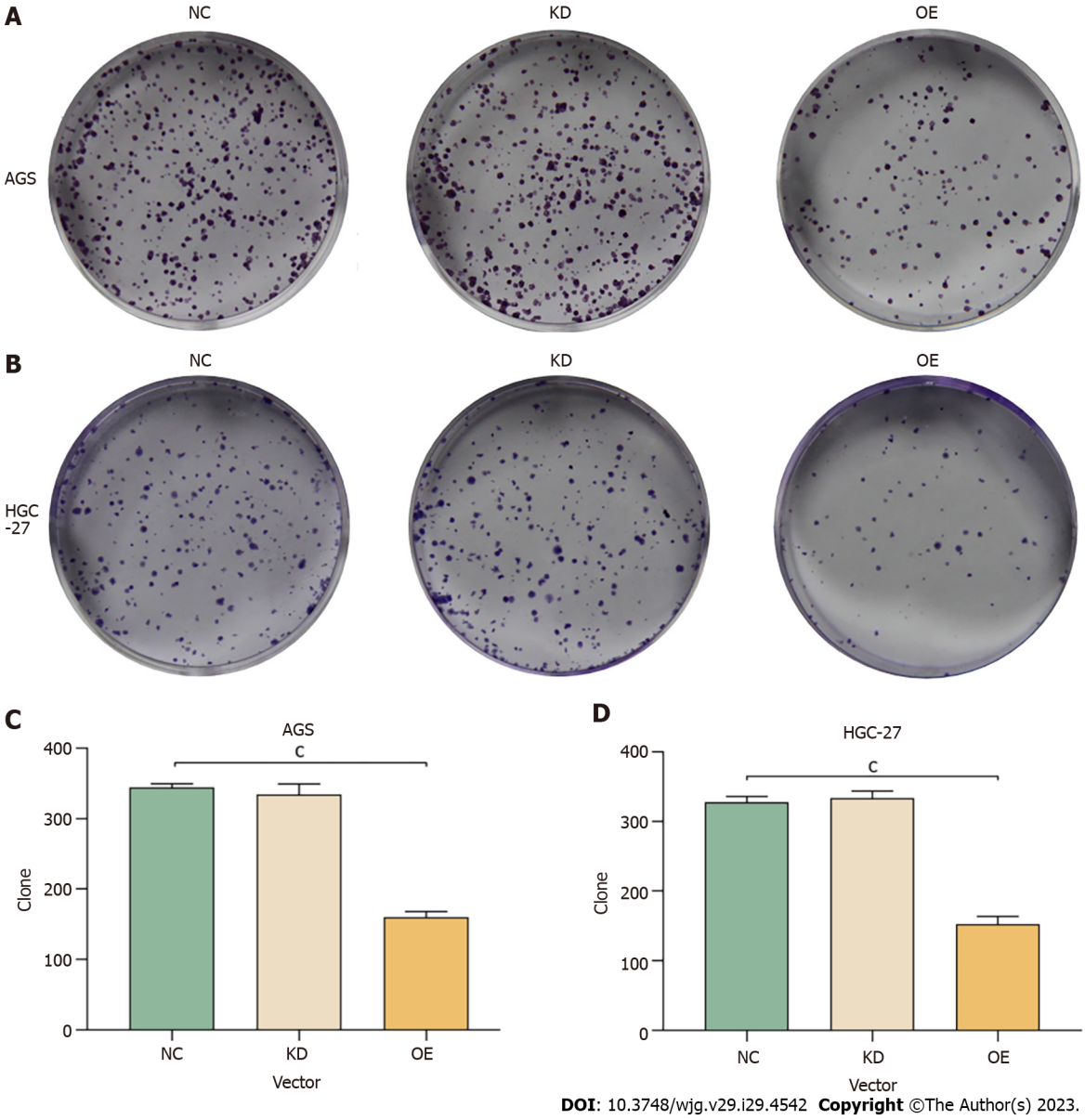

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Figure 3 Effect of miR-204-3p on colony forming ability in AGS and HGC-27 cells. A and B: Crystal violet staining diagram of AGS and HGC-27 cells; C and D: Statistical results of colony forming ability in AGS and HGC-27 cells. °P < 0.001. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

MiR-204-3p retarded GC cell cycle

We analyzed cell cycle distribution to investigate whether miR-204-3p inhibited GC cell proliferation by mediating the cell cycle (Figure 5). Among AGS cells, the G0/G1 phase percentages were 44.91 ± 1.15%, 45.36 ± 0.70% and 49.30 ± 0.41% in the NC, KD and OE groups, respectively (Figure 5A and C). Among HGC-27 cells, the G0/G1 phase percentages were 29.36 ± 0.29%, 29.57 ± 1.11% and 41.03 ± 0.47% in the NC, KD and OE groups, respectively (Figure 5B and D). These results revealed that AGS and HGC-27 cells with miR-204-3p overexpression had a notably higher number of cells in G0/G1 phase, which indicated that miR-204-3p overexpression blocked GC cell cycle in the G0/G1 phase.

MiR-204-3p inhibited the formation of subcutaneous tumor in BABL/c nude mice

A subcutaneous tumor formation experiment was conducted in BABL/c nude mice using lentivirus-transfected MKN-45 cells to observe the changes in tumor size and body weight. The results revealed that the OE group had smaller tumors compared to the NC group (Figure 6A and B). According to tumor growth data, it was found that back tumor growth was notably slower in the OE group compared to the NC group (Figure 6C). Additionally, the weight of BABL/c nude mice in the OE group increased significantly (Figure 6D), which indicated that miR-204-3p overexpression suppressed subcutaneous tumorigenesis in BABL/c nude mice.

Effect of miR-204-3p on the MAPK signaling pathway

We investigated the effect of miR-204-3p on the MAPK signaling pathway. Firstly, the impact of miR-204-3p on the

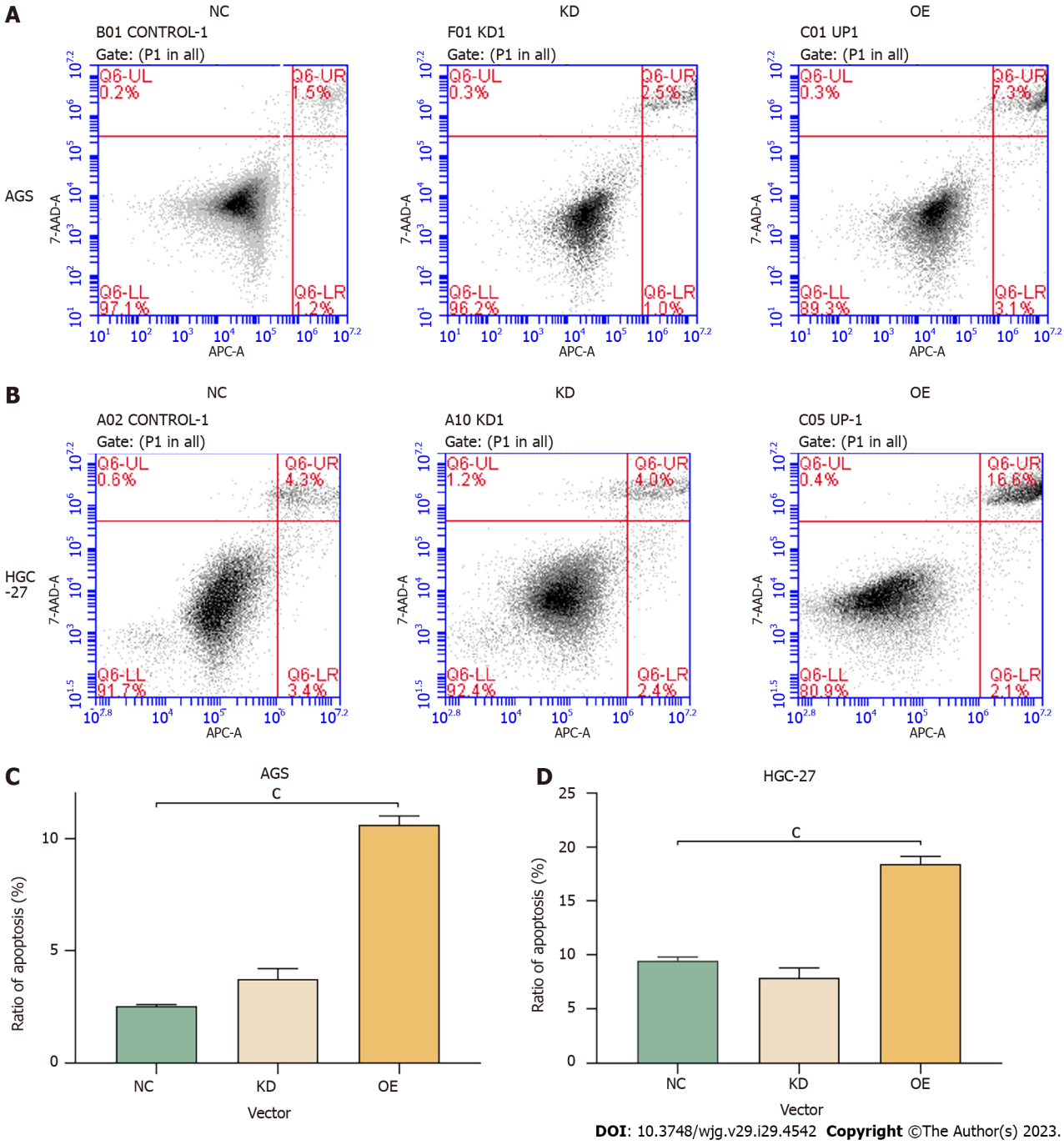

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Figure 4 Effects of miR-204-3p on apoptosis in AGS and HGC-27 cells. A and B: Flow cytometry was used to analyze apoptosis of AGS and HGC-27 cells; C and D: Statistical results of AGS and HGC-27 cell apoptosis. °$P < 0.001$. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

mRNA levels of *KRAS*, *ERK1* and *ERK2* was detected using qRT-PCR. The results revealed that miR-204-3p overexpression resulted in a significant decrease in *KRAS*, *ERK1* and *ERK2* in AGS and HGC-27 cells (Figure 7A and B). We further investigated the impact of miR-204-3p on the MAPK signaling pathway-related proteins, including KRAS, ERK1/2, and p-ERK1/2. The results showed that in HGC-27 and AGS cells, miR-204-3p overexpression caused noteworthy lower levels of KRAS and p-ERK1/2, but no significant difference was observed in ERK1/2 (Figure 7C-F). These findings indicated that miR-204-3p overexpression effectively inhibited GC cell proliferation, and this effect was achieved through the inhibition of KRAS, which subsequently prevented the phosphorylation of downstream effector protein ERK1/2 in the MAPK signaling pathway.

Effects of miR-204-3p on apoptosis-related proteins

The effect of miR-204-3p on apoptosis-related proteins in GC cells was investigated. The results indicated a reduction in BCL-2 and the BCL-2/BAX ratio, as well as an increase in Caspase-3 in the OE group (Figure 8A-D), which confirmed that miR-204-3p overexpression stimulated GC cell apoptosis *via* the BCL-2/BAX signaling pathway.

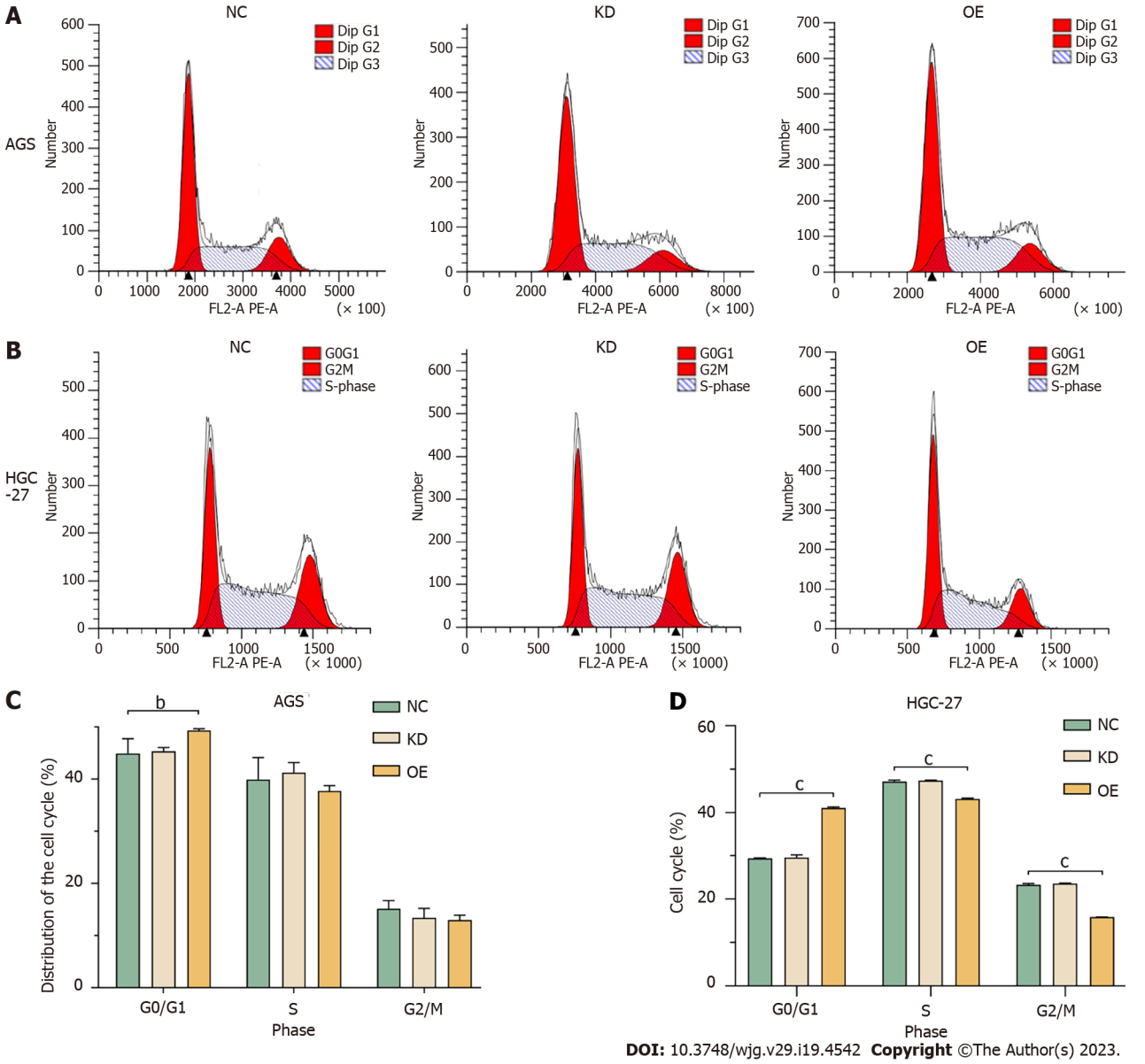

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Figure 5 Effects of miR-204-3p on the cell cycle in AGS and HGC-27 cells. A and B: Cell cycle analysis in AGS and HGC-27 cells by flow cytometry; C and D: Percentage of cell cycle distribution in AGS and HGC-27 cells. $^{b}P < 0.01$, $^{c}P < 0.001$. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

Effect of miR-204-3p on necroptosis-related proteins

We examined the protein changes in RIP1, p-RIP1, MLK1 and p-MLK1 during necroptosis and investigated the impact of miR-204-3p on GC cells necroptosis. The results revealed that miR-204-3p overexpression significantly inhibited the protein expression levels of p-RIP1 and p-MLK1 (Figure 8E-H), which confirmed that miR-204-3p overexpression inhibited GC cells necroptosis *via* the RIP1/MLK1 signaling pathway.

DISCUSSION

MiR-204-3p plays a crucial role in various diseases. Some studies have confirmed that miR-204-3p upregulation can be targeted to inhibit Nox4 to reduce memory deficits[21]. Moreover, its overexpression inhibited high glucose induced lens epithelial cells migration and epithelial-mesenchymal transition (EMT), and inhibited high glucose induced podocytes apoptosis and dysfunction[13,15]. Notably, miR-204-3p upregulation inhibited malignant melanoma migration, invasion and EMT progression by targeting inhibition of PAX2[19], its upregulation also inhibited hepatocellular carcinoma cell proliferation by targeting inhibition of FN1[22]. MiR-204-3p was found to be underexpressed in bladder cancer tissues and was related to poor prognosis, it regulated bladder cancer cell proliferation by targeting LDHA mediated glycolysis [16]. Apoptosis of glioma cells is induced by miR-204-3p targeting IGFBP2[23]. Furthermore, LINC00963 was overexpressed in osteosarcoma tissues and was related to poor prognosis, miR-204-3p reversed LINC00963 in promoted

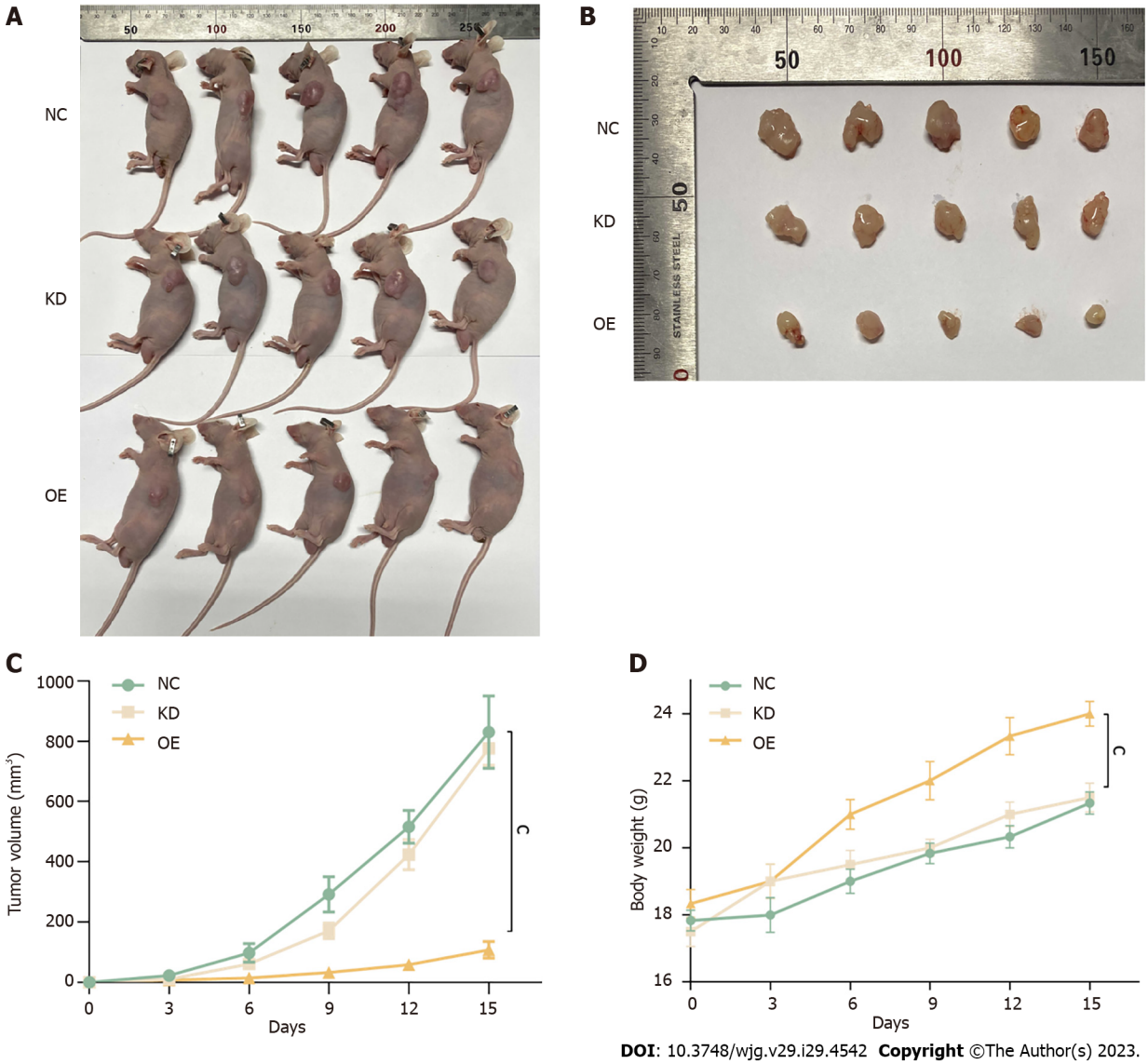

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Figure 6 Effect of miR-204-3p on tumor size in BABL/c nude mice. A: Comparison of gastric carcinoma tumor xenograft models in BABL/c nude mice; B: Comparison of subcutaneous tumors on the back in BABL/c nude mice; C: Statistical graph of tumor growth curve for gastric carcinoma tumor xenograft models in BABL/c nude mice; D: Statistical graph of body weight growth curve in BABL/c nude mice. $^{c}P < 0.001$. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

osteosarcoma cell proliferation and inhibited migration and invasion[24]. LINC00514 was upregulated in GC tissues, its overexpression stimulated GC cell growth and inhibited EMT by sponging miR-204-3p/KRAS[20]. These results suggest that miR-204-3p may be a new target for cancer therapy.

Firstly, we found that the expression of miR-204-3p was low in GC cells, and its overexpression resulted in the inhibition of cell proliferation, colony formation ability, and the cell cycle, while promoting apoptosis. *In vivo* tumor formation experiments in 4-week-old BABL/c nude mice verified that miR-204-3p overexpression inhibited subcutaneous tumor growth. Thus, both *in vitro* and *in vivo* experiments demonstrated the inhibitory influence of miR-204-3p overexpression on GC cells.

Apoptosis, a programmed cell death, is a natural barrier against tumorigenesis. However, in cancer, abnormal expression of anti-apoptotic or pro-apoptotic proteins causes inhibition of apoptotic pathways[25]. For instance, BCL-2 functions as an anti-apoptotic protein, preventing cytochrome C liberation from mitochondria, thereby inhibiting apoptosis. On the other hand, BAX is a common pro-apoptotic protein that can form homodimers or bind to BCL-2 to form heterodimers that activate Caspase-3 to promote apoptosis[26]. The BCL-2/BAX ratio is closely related to tumor progression, and a lower ratio indicates a stronger apoptosis effect. Therefore, most anti-cancer drugs rely on the BCL-2/BAX mechanism to kill cancer cells[27]. This study verified that miR-204-3p overexpression led to the upregulation of Caspase-3 in GC cells, while downregulating BCL-2 and the BCL-2/BAX ratio. These findings provide evidence that miR-204-3p overexpression can stimulate apoptosis and inhibit GC cell proliferation through the BCL-2/BAX/Caspase-3 apoptosis pathway.

In tumors, the MAPK pathway is frequently activated to control apoptosis, cell growth, and cell division[28]. Signal transmission of the MAPK signaling pathway follows a three-step enzyme-linked reaction. KRAS, as an upstream

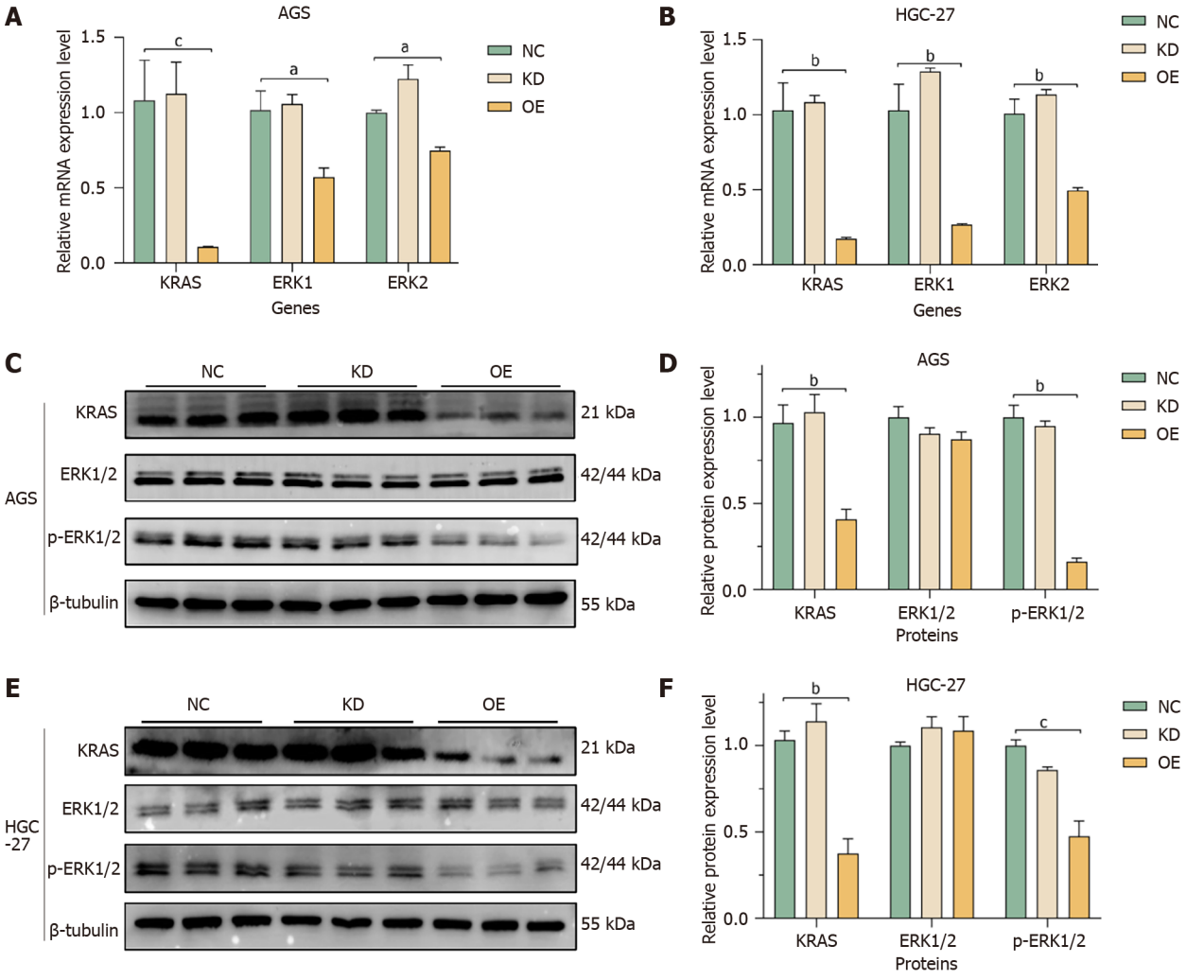

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Figure 7 Effect of miR-204-3p on the MAPK signaling pathway in AGS and HGC-27 cells. A and B: Effects of miR-204-3p on mRNA levels of *KRAS*, *ERK1* and *ERK2* in AGS and HGC-27 cells; C and D: Effects of miR-204-3p on protein levels of *KRAS*, *ERK1/2* and *p-ERK1/2* in AGS cells; E and F: Effects of miR-204-3p on protein levels of *KRAS*, *ERK1/2* and *p-ERK1/2* in HGC-27 cells. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

activation protein, is activated when bound to GTP. This change causes the recruitment of KRAS to RAF on the cell membrane and promotes RAF activation. Activated RAF phosphorylates and activates MEK, while MEK phosphorylates and further activates ERK, which is located at the end of the signaling pathway and can transfer into the nucleus and bind to transcription factors, thereby regulating transcription programs and mediating cell growth, migration and differentiation[29-31]. Previously, we established that miR-204-3p targeted KRAS[20]. The current study confirmed that miR-204-3p upregulation can inhibit KRAS and p-ERK1/2, which suggested that miR-204-3p overexpression could inhibit the MAPK signaling pathway.

Necroptosis is a newly discovered mechanism of programmed cell death that has the potential to regulate tumorigenesis[32]. This process is primarily regulated by three proteins: RIP1, RIP3 and MLK1. Specifically, RIP1 is activated through phosphorylation, which then recruits RIP3[33]. Once activated, phosphorylated RIP3 can oligomerize MLK1 and transfer it to the plasma membrane, ultimately resulting in necroptosis characterized by cell swelling and organelle damage[34,35]. Interestingly, necroptosis has been found to both promote and inhibit cancer growth. As a form of cell death, necroptosis inhibits the development of tumors, yet it may also incite an inflammatory reaction that encourages cancer metastasis and immunosuppression. Research has revealed that glioblastoma, pancreatic cancer, and lung cancer can be impacted by the upregulation of RIP1, RIP3, and MLK1[36-38]. It was found that downregulation of MLK1 inhibited tumor cell growth and increased sensitivity to radiotherapy in both GC and ovarian cancer[39,40]. We detected necroptosis-related proteins and discovered that miR-204-3p overexpression decreased p-RIP1 and p-MLK1. These findings suggest that miR-204-3p overexpression can inhibit necroptosis through the RIP1/MLK1 pathway, ultimately inhibiting GC cell proliferation.

To sum up, our study verified that miR-204-3p is underexpressed in GC, and that its overexpression inhibits GC cell proliferation, promotes apoptosis, arrests the cell cycle in the G0/G1 phase, inhibits cell colony formation and the formation of subcutaneous tumors. Necroptosis is typically initiated by tumor necrosis factor (TNF) stimulation[41]. RIP1 binds to FADD, which then recruits caspase-8. The activation of caspase-8 promotes the process of RIP1-dependent

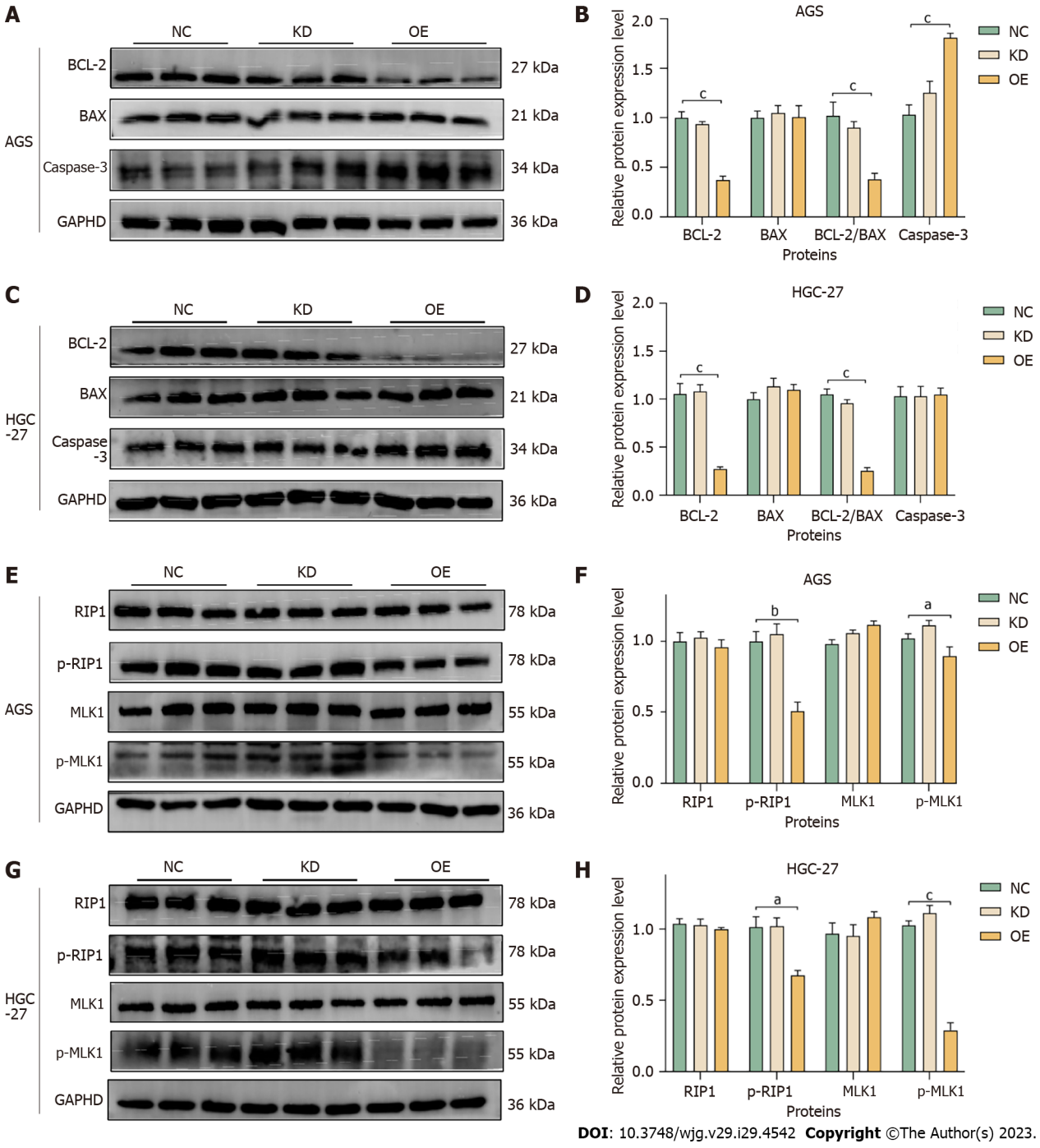

Figure 8 Effects of miR-204-3p on apoptosis-related proteins and necroptosis-related proteins in AGS and HGC-27 cells. A and B: Effect of miR-204-3p on apoptosis-related proteins BCL-2, BAX and Caspase-3 in AGS cells; C and D: Effect of miR-204-3p on apoptosis-related proteins BCL-2, BAX and Caspase-3 in HGC-27 cells; E and F: Effects of miR-204-3p on necroptosis-related proteins RIP1, p-RIP1, MLK1, and p-MLK1 in AGS cells; G and H: Effects of miR-204-3p on necroptosis-related proteins RIP1, p-RIP1, MLK1, and p-MLK1 in HGC-27 cells. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$. NC: Empty lentiviral vector; KD: miR-204-3p knockdown lentiviral vector; OE: miR-204-3p overexpression lentiviral vector.

apoptosis[32]. Additionally, RIP1 activates ERK to regulate the MAPK signaling pathway[42-44]. In the MAPK signaling pathway, phosphorylation of ERK can activate BCL-2, which in turn stimulates the apoptosis pathway and accelerates the process of apoptosis[45,46]. We verified that miR-204-3p overexpression can inhibit GC cell proliferation by inhibiting the MAPK signaling pathway and inhibiting the RIP1/MLK1 necroptosis pathway to promote the BCL-2/BAX/Caspase-3 apoptosis pathway (Figure 9).

CONCLUSION

MiR-204-3p overexpression inhibited GC cell proliferation by inhibiting the MAPK pathway and necroptosis pathway to

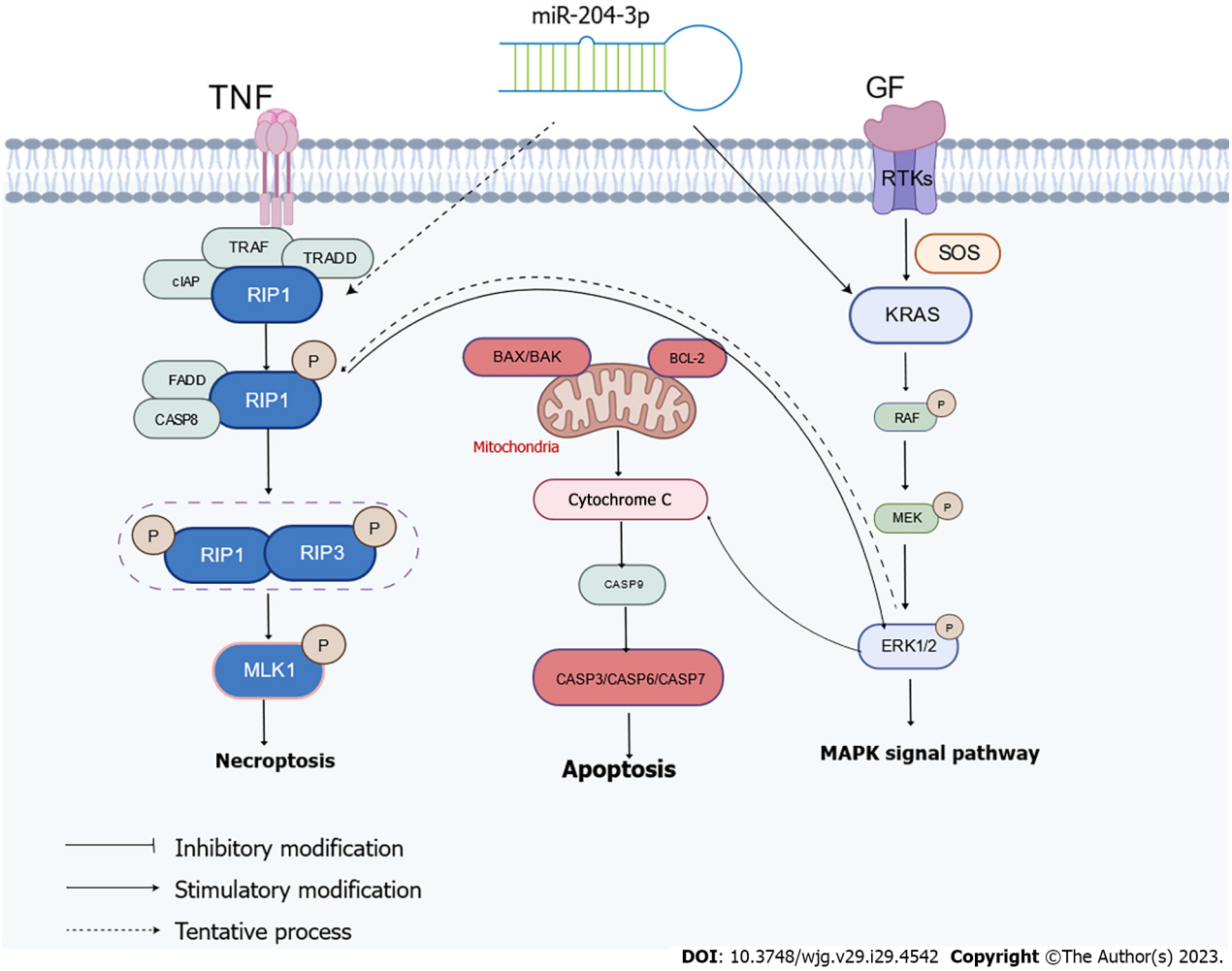

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Figure 9 Mechanism of the effect of miR-204-3p on the MAPK signaling pathway, apoptosis and necroptosis. TNF: Tumor necrosis factor; GF: Germ-free.

promote GC cell apoptosis. Thus, miR-204-3p may represent a new potential therapeutic target for GC.

ARTICLE HIGHLIGHTS

Research background

Gastric carcinoma (GC) is a common gastrointestinal malignancy worldwide. Based on the cancer-related mortality, the current prevention and treatment strategies for GC still show poor clinical results. Therefore, it is important to find effective treatment targets.

Research motivation

At present, the main treatment for GC is surgery, chemotherapy and radiotherapy, but the therapeutic effect is not ideal.

Research objectives

To explore the effect of miR-204-3p on GC cells.

Research methods

We determined the expression level of miR-204-3p in GC, and then used an miR-204-3p overexpression vector and an miR-204-3p knockdown vector in GC cells. The influence of miR-204-3p on the changes in cell phenotype and tumorigenicity *in vivo* was assessed. Furthermore, the effects of miR-204-3p on various proteins related to the MAPK signaling pathway, necroptosis signaling pathway and apoptosis signaling pathway in GC cells were investigated.

Research results

It was found that miR-204-3p was underexpressed in GC, and miR-204-3p overexpression inhibited GC cell viability, promoted cell apoptosis, blocked the cell cycle, inhibited colony formation ability and inhibited tumorigenicity *in vivo*. It

was also verified that miR-204-3p overexpression can promote apoptosis by inhibiting the MAPK pathway and the necroptosis pathway, thus inhibiting GC cell proliferation.

Research conclusions

MiR-204-3p overexpression inhibited GC cell proliferation by inhibiting the MAPK pathway and the necroptosis pathway to promote GC cell apoptosis.

Research perspectives

MiR-204-3p can be used for targeted therapy of GC, and can also be used as a new biomarker for GC.

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FOOTNOTES

Author contributions: Li X carried out most of the studies, analyzed the data, and wrote the manuscript; Nan Y designed the study and revised the manuscript; Tibenda JJ, Du YH and Huang SC wrote the manuscript, and conducted the chart-making work; Ning N and Chen GQ were responsible for the total transcriptomic; Yang YT and Meng FD performed parts of the *in vivo* and *in vitro* experiments, and conducted statistical analysis of the data; Yuan L and Nan Y provided the conceptual and technical guidance and revised the manuscript critically for important intellectual content; all authors have read and approved the manuscript.

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ORIGINAL ARTICLE

Basic Study

Effect of exogenous hydrogen sulfide in the nucleus tractus solitarius on gastric motility in rats

Hong-Zhao Sun, Chen-Yu Li, Yuan Shi, Jin-Jin Li, Yi-Ya Wang, Li-Na Han, Lu-Jie Zhu, Ya-Fei Zhang

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Abstract

BACKGROUND

Hydrogen sulfide (H_2S) is a recently discovered gaseous neurotransmitter in the nervous and gastrointestinal systems. It exerts its effects through multiple signaling pathways, impacting various physiological activities. The nucleus tractus solitarius (NTS), a vital nucleus involved in visceral sensation, was investigated in this study to understand the role of H_2S in regulating gastric function in rats.

AIM

To examine whether H_2S affects the nuclear factor kappa-B (NF-κB) and transient receptor potential vanilloid 1 pathways and the neurokinin 1 (NK1) receptor in the NTS.

METHODS

Immunohistochemical and fluorescent double-labeling techniques were employed to identify cystathionine beta-synthase (CBS) and c-Fos co-expressed positive neurons in the NTS during rat stress. Gastric motility curves were recorded by inserting a pressure-sensing balloon into the pylorus through the stomach fundus. Changes in gastric motility were observed before and after injecting different doses of NaHS (4 nmol and 8 nmol), physiological saline, Capsazepine (4 nmol) + NaHS (4 nmol), pyrrolidine dithiocarbamate (PDTC, 4 nmol) + NaHS (4 nmol), and L703606 (4 nmol) + NaHS (4 nmol).

RESULTS

We identified a significant increase in the co-expression of c-Fos and CBS positive neurons in the NTS after 1 h and 3 h of restraint water-immersion stress compared to the expressions observed in the control group. Intra-NTS injection of NaHS at different doses significantly inhibited gastric motility in rats ($P < 0.01$). However, injection of saline, first injection NF-κB inhibitor PDTC or transient receptor potential vanilloid 1 (TRPV1) antagonist Capsazepine or NK1 receptor blockers

L703606 and then injection NaHS did not produce significant changes ($P > 0.05$).

CONCLUSION

NTS contains neurons co-expressing CBS and c-Fos, and the injection of NaHS into the NTS can suppress gastric motility in rats. This effect may be mediated by activating TRPV1 and NK1 receptors *via* the NF-κB channel.

Key Words: Nucleus tractus solitarius; Hydrogen sulfide; Gastric motility; Nuclear factor kappa-B; Transient receptor potential vanilloid 1

Core Tip: This study revealed a significant inhibitory effect of exogenous hydrogen sulfide on gastric motility in rats. This effect appeared to involve the release of substance P, potentially activating the transient receptor potential vanilloid 1 pathway mediated by nuclear factor kappa-B channels.

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INTRODUCTION

The number of patients with stress-induced gastric ulcers has increased dramatically[1], and stress is highly associated with several functional gastrointestinal disorders, such as functional dyspepsia and irritable bowel syndrome[2]. The nucleus tractus solitarius (NTS) is a relay nucleus for visceral primary afferent neural signaling. It receives sensory afferents from visceral organs and projects to the spinal cord to regulate respiratory and cardiovascular activity. The NTS is also closely connected with various brain nuclei[3]. Recent studies have demonstrated the role of the NTS in cardiovascular and respiratory regulation and the reflex regulation of intragastric pressure. Synapses mediate the vagal-dependent gastric reflex between vagal afferent fibers and NTS neurons, and through the vagal preganglionic parasympathetic neurons in the dorsal vagal complex[4]. Neuronal firing studies in the NTS have shown that H_2S increases NTS-evoked postsynaptic currents by enhancing presynaptic glutamate release and affects the membrane potential of NTS neurons in a concentration-dependent manner[5].

Vagal afferent transmission primarily terminates in the NTS[6], which may act as a relay activator to inhibit spinal neuronal activity. Vagal-mediated glutamate release can regulate homeostasis by activating NTS neurons and metabotropic glutamate receptors, contributing to homeostatic regulation. Injection of oxytocin in the NTS has been observed to inhibit gastric smooth muscle diastole in rats, possibly through the cAMP-PKA signaling pathway[7]. The microinjection of monosodium glutamate in the NTS and the activation of NTS opioid receptors inhibit gastric motility and significant c-Fos expression in the dorsal motor nucleus of the vagus (DMV) neurons[8].

However, hydrogen sulfide (H_2S) is a recently discovered gas transmitter endogenously produced in the human and animal brain and organ tissues. Cystathionine beta-synthase (CBS) mainly synthesizes H_2S in the central nervous system and plays a significant physiological role[9] and has a protective role in neurodegenerative diseases such as Parkinson's disease[10] and Alzheimer's disease[11]. CBS is present in neurons and glial cells of the NTS, exerting excitatory effects and modulating synaptic neuronal activity[12]. Blocking CBS attenuates synaptic transmission in NTS neurons. Applying the H_2S donor NaHS also enhances synaptic transmission in NTS neurons. H_2S in the NTS plays an equally important role as a gaseous neuromodulator in maintaining or modulating autonomicand other systems[13]. In vitro experiments have shown that H_2S relaxes gastrointestinal smooth muscles, inhibiting spontaneous movements and responses to chemical or electrical stimuli[14]. H_2S also plays a role in other types of smooth muscle relaxation *via* K^+-ATP channels[15], suggesting that endogenous H_2S has a regulatory role in the gastrointestinal tract's motor function.

The nuclear factor kappa-B (NF-κB) pathway is activated by various factors and plays a crucial role in the immune response and inflammation. A study found that NaHS administration in the rat intraperitoneal *via* sulfhydration caused NF-κB activation and lung inflammation, a significant increase in p65 protein levels, vascular congestion, and neutrophil infiltration. Also, slight neuronal degeneration was observed in the rat heart, liver, and brain, suggesting that H_2S acts on NF-κB channels for messaging[16]. H_2S also interacts with nitric oxide to cause vasodilation, down-regulates NF-kB pathway-induced inflammation, fibrosis and damage from prolonged or intense oxidative stress; protects tissues from ischemia- and reperfusion-induced injury; and reduces immune rejection by reducing oxygen free radicals produced *in vivo*[17]. Additionally, H_2S demonstrates antioxidant and anti-apoptotic effects on neurons and glial cells[18].

Transient receptor potential vanilloid type 1 (TRPV1) is a non-selective calcium channel associated with nociceptive sensations in peripheral nerves. Its activation can lead to neurokinin 1 (NK1) receptor activation, pain, local neurogenic inflammation, and systemic anti-inflammatory/analgesic effects, and enhanced transmitter release in the NTS. TRPV1 involves various physiological and pathological processes[19,20]. Electrophysiological studies have shown that the

activation of TRPV1 by capsaicin enhances glutamate release to visceral sensory neurons, affecting NTS preganglionic neurons[21]. H_2S can cause peripheral inflammation and synaptic enhancement of glutamatergic signaling in the spinal cord by activating TRPV1 channels, thus stimulating other receptors at the terminals of capsaicin-sensitive neurons[22]. TRPV1 activation also leads to the release of substance P (SP), while NK1 receptors are responsible for neurally mediated digestive secretion and contributes to brain homeostasis and sensory neuronal transmission associated with depression, stress, anxiety and vomiting[23]. H_2S causes gastric juice secretion by stimulating TRPV1 receptors on primary afferent nerve fibers and modulates cholinergic neurons by releasing SP to act on NK1, NK2, or NK3 receptors[24]. Therefore, we speculate that TRPV1 channels may be involved in the effect of H_2S donors on gastric emptying.

SP is a neuroactive peptide involved in pain and inflammation[25]. It is widely present in the mammalian organism in the central, peripheral, and gastrointestinal nervous systems[26] and other tissues, participating in various physiopathological processes, including stress, emotional anxiety, and immune regulation. NK1 receptors are the primary receptors for SP and are widely expressed in the brain, contributing to stress and emotional anxiety[27]. SP is widely expressed in the NTS, mainly in the primary sensory neurons in the peripheral nervous system, and intrinsic neurons of the gastrointestinal tract. It has been shown to have neurotransmitter effects in the central and peripheral nervous systems and is associated with immune and inflammatory diseases of the respiratory and gastrointestinal systems[28].

Herein, we investigated the regulatory effects of H_2S in the NTS on rat gastric function and explore whether these effects were mediated by SP release *via* NF-κB channel-dependent activation of the TRPV1 pathway.

MATERIALS AND METHODS

Animal

The animals used in this experiment were 240-280 g adult male Wistar rats purchased from Jinan Panyue Experimental Animal Breeding Co and housed in separate cages at a constant temperature (22 °C ± 2 °C) given appropriate food and water based on their body weight. To allow them to acclimate to their surroundings, the rats were exposed to natural rhythmic light for one week before the start of the experiment.

Before the experiment, the rats underwent a 24-h fasting period, during which they were allowed to freely drink. The other environmental conditions remained consistent throughout the experiment. The Experimental Animal Ethics Committee of Qilu Normal University approved the experiment. All experiments complied with internationally accepted ethical standards. The study also adhered to the guidelines set by the International Association for the Study of Pain[29].

Chemicals

NaHS, L703606, PDTC, Capsazepine, and protamine sky blue were purchased from Sigma-Aldrich (St. Louis, MO, United States). NaHS was dissolved in 0.9% saline, while the other chemicals were dissolved in dimethyl sulfoxide and reconstituted in saline. For the immunohistochemical fluorescence double labeling, the following reagents were purchased from Servicebio (Wuhan, China): Goat serum, anti-CBS rabbit pAb, FITC-conjugated goat anti-rabbit IgG, Cy3-conjugated goat anti-mouse IgG, and anti-c-Fos mouse pAb.

Immunohistochemical fluorescence double labeling

We used the restraint water immersion stress model to investigate acute stress-induced gastric mucosal injury in rats. This acute compound stress model causes changes in gastric function in rats under stress through enhanced parasympathetic activity in the innervated stomach[30]. Once anesthetized, the rats were swiftly removed from the bottle and secured to a wooden board using medical tape to immobilize their limbs and teeth. When awake, the rats were then immersed in cold water (21 °C ± 1 °C) with the sternal process aligned with the water level. To minimize experimental error, consistent time points were selected for each experiment.

The rats were randomly divided into three groups (n = 6) based on the duration of restraint water-immersion stress (RWIS) (0 h, 1 h, or 3 h). Cardiac perfusion was performed using 500 mL of prepared 0.01 mol/L phosphate-buffered saline (PBS) followed by 500 mL of 4% 0.1 mol/L paraformaldehyde (PFA). After administering an overdose of isoflurane to sacrifice the rat, the thoracic cavity was opened along the sternal process, and the heart was exposed. The infuser needle was inserted into the heart's left ventricle, securing the heart, while the right auricle was incised to allow blood to drain. The rat's liver was flushed with 0.01 mol/L PBS buffer until it turned white, followed by perfusion with 4% PFA solution using a "fast and then slow" principle, gradually reducing the flow rate when the rat's limbs twitched.

Upon completion of perfusion, the rat's head was severed, and the brain was extracted. The brain was placed in a small wide-mouth flask containing 4% PFA and kept at 4 °C for 24 h. Subsequently, the fixed rat brain was transferred to a 0.1 mol/L 30% sucrose solution for dehydration. The frozen target nuclei region was then sectioned into 30 μm thick coronal sections using a sectioning machine and stored in 0.01 mol/L PBS.

Next, each well of a multi-well plate was filled with 500 μL of 0.01 mol/L PBS buffer to clean the brain slices and remove impurities. A methanolic solution of 3% H_2O_2 was added to block endogenous peroxidase activity. The wells were then incubated with a goat serum closure solution for 1 h to enhance cell membrane permeability. Subsequently, 500 μL of each primary antibody working solution was added, consisting of mouse anti-c-Fos (diluted at 1:500) and rabbit anti-CBS (diluted at 1:500), and incubated overnight at 4 °C.

Finally, 500 μL of each fluorescent secondary antibody working solution was added for 1 h. Any residual fluorescent secondary antibody was washed off with PBST. Previously treated with chromium-vanadium gelatin, the brain slides were placed on glass slides and allowed to dry naturally. The slides were sealed with an anti-fluorescence quencher, ensuring the removal of air bubbles using a vacuum. Finally, the sealed fluorescent glass slide was placed under an

Olympus Fluorescence confocal microscopy to observe and compare the brain atlas to determine the position of the NTS, observe the CBS and c-Fos-positive neurons number, and take pictures. The expression of c-Fos and CBS in the NTS was counted using Image Pro-Plus 6.0 software (Number/0.01 mm^2).

Experimental grouping

We evaluated the following subgroups to investigate the regulatory effects of NaHS in the NTS on gastric function and its underlying mechanisms. The chosen doses were based on pre-experiments and relevant literature[31]: (1) The effect of microinjection of NaHS (0.1 μL, 4 nmol; n = 6) in NTS on gastric motility; (2) the effect of microinjection of NaHS (0.1 μL, 8 nmol; n = 6) in NTS on gastric motility; (3) the effect of microinjection of saline (0.1 μL; n = 6) in NTS on gastric motility as a control group; (4) the effect of microinjection of NaHS (0.1 μL, 4 nmol) + PDTC (0.1 μL; n = 6) in NTS on gastric motility; (5) microinjection of NaHS (0.1 μL, 4 nmol) + Capsazepine (0.1 μL, 4 nmol; n = 6) in NTS on gastric motility; and (6) microinjection of NaHS (0.1 μL, 4 nmol) + L703606 (0.1 μL, 4 nmol; n = 6) in NTS on gastric motility.

Microinjection

Before conducting the experiments, the rats were anesthetized with 4% chloral hydrate at (400 mg/kg body weight) by intraperitoneal injection until the eyelids and corneal reflexes disappeared, the muscles were relaxed, and the breathing was uniform. The anesthetized rat head was fixed on a brain stereotaxic apparatus (Stoelting 68002, Shenzhen Ruiwode Company, China).

Next, the animal was fixed according to the rat brain stereotaxic atlas (Paxinos and Watson, 2007) using the three points of the animal's bilateral inner ear holes and incisors. With the left and right ear rods reading the same, fontanelle and bregma were kept at the same level with an error of no more than 0.3 mm.

The head hair was removed with hair clippers to expose the scalp and disinfected with 75% alcohol. Then, the scalp was cut along the sagittal suture of the skull with ophthalmic clippers to expose the skull, the excess connective tissue around the skull was cut away, and the surface of the skull was gently wiped with saline until the fontanelle and herringbone suture were exposed. The three-dimensional coordinates of fontanelle were used as the zero point. A small hole of approximately 2 mm in diameter was drilled in the skull ipsilateral to the coordinates of the NTS center point (13.3 mm posterior to fontanelle, 0.8 mm paracentral opening, 7.9 mm subdural) in the atlas using an electric cranial drill. A glass microelectrode with a tip of approximately 30 μm was placed into the brain at the depth of the coordinates.

Recording gastric motility

The anesthetized rats were placed abdomen side up, and a small incision was made in the fundus of the stomach to clean the gastric residue. A 5 mm diameter balloon filled with warm water was inserted into the pylorus of the gastric sinus and kept at a constant baseline pressure. The balloon inserted into the rat's stomach was connected to the pressure transducer and BL-420 (Biological Function Experimental System; Chengdu Taimeng Company, China) *via* a polyethylene plastic tube. The stimulation parameters of the transducer were adjusted to 25 mm/min speed, 0.5 mV/cm sensitivity, and 10 Hz filter. Once the gastric motility curve was stabilized, the drug's microinjections and contaminate sky blue were administered. A heat lamp was used throughout the experiment to maintain a constant ambient temperature, and gastric motility was recorded.

Histological identification of the microinjection site

After gastric motility recording, 2% pontamine sky blue (0.1 μL) was injected into the NTS, the rats were executed with an excess of sodium pentobarbital, and the thorax was opened for cardiac perfusion. After perfusion, the heads of the rats were cut off, and the brain tissues were removed and placed in a 4% formaldehyde solution for fixation.

Subsequently, the brain tissues were frozen at -16 °C for 30 min and sectioned into successive coronal sections with a thickness of 16 μm. The brainstem sections were stained, allowing for the identification of injection sites. The brain slices on slides were treated with a neutral red stain and dehydrated to achieve transparency. The sections were observed and photographed using a microscope (Nikon Optiphot, Nikon, Shanghai, China) and photographed with a digital camera (Magnafire; Optronics, Goleta, CA, United States) connected to a computer. The blue dot marking the precise location of the NTS was identified for further statistical analysis.

Data analysis and statistics

The gastric motility of the rats was assessed by counting the number of contractions before and 5 min after injection were counted respectively. The total duration of contraction waves (T.D.C.W) within 5 min, the total amplitude of contraction waves (T.A.C.W) within 5 min and the gastric motility index (the product of amplitude and duration) before and after the 5-min microinjection were evaluated statistically. To calculate the inhibition rate of gastric motility, the following formula was used: Inhibition rate (%) = (pre-injection value-post-injection value) × 100%/pre-injection value. The height between the highest point of the contraction curve and the baseline is the amplitude of the contraction wave. The time duration between the starting point and the ending point of the contraction wave is the time duration of the contraction wave.

Statistical analysis was performed using SPSS v25.0 (IBM SPSS Inc., Chicago, IL, United States) using Student's t-test or one-way ANOVA, followed by a posthoc test using the Student-Newman-Keuls test. All data are presented as mean ± SE. A P value less than 0.05 was considered statistically significant.

RESULTS

Observation of c-Fos and CBS expression in NTS at different times in RWIS

In this experiment, the number of CBS and c-Fos co-expressing neurons in the NTS (Figure 1, n = 6) was revealed by immunohistochemical fluorescence double-labeling. The expression of c-Fos protein in the NTS showed varying degrees of increase at 1 h (7.00 ± 0.37) and 3 h (4.83 ± 0.31) after RWIS compared to the control group at 0 h (1.83 ± 0.30) (P < 0.01). This finding indicates that CBS neurons in the NTS of rats were activated during the RWIS procedure.

NTS injection site identification

Following neutral red staining, the brain slices were examined under a light microscope to determine the localization of the injected blue spots and drugs within the NTS. The observed data regarding the gastric motility of rats in the correct position were analyzed. Figure 2 presents the diagram for identifying the degree of tissue localization.

NaHS inhibits gastrointestinal motility within NTS

The microinjection of physiological saline (PS) (0.1 μL, n = 6) under the same conditions did not produce a significant change in gastric motility (Figure 3A). In contrast, the microinjection of NaHS at different concentrations (4 nmol and 8 nmol, 0.1 μL, n = 6) into the rat NTS resulted in significant inhibition of gastric motility (Figure 3B and C).

We compared gastric motility curves measured before and 5 min after the drug injection and after 4 nmol NaHS injection in the NTS. The T.A.C.W. decreased from 553.08 mm 5 min^{-1} ± 9.59 mm 5 min^{-1} to 421.30 mm 5 min^{-1} ± 10.58 mm 5 min^{-1} (P < 0.01). The T.D.C.W. decreased from 179.79 s 5 min^{-1} ± 13.33 s 5 min^{-1} to 132.56 s 5 min^{-1} ± 6.67 s 5 min^{-1} (P < 0.01), and the gastric motility index (G.M.I.) decreased from 5219.88 ± 182.11 to 4250.28 ± 159.03 (P < 0.01). At a NTS microinjection dose of 8 nmol NaHS, the T.A.C.W. decreased from 587.62 mm 5 min^{-1} ± 9.58 mm 5 min^{-1} to 407.44 mm 5 min^{-1} ± 10.61 mm 5 min^{-1} (P < 0.01) and the T.D.C.W. decreased from 234.11 s 5 min^{-1} ± 11.74 s 5 min^{-1} to 145.13 s 5 min^{-1} ± 3.93 s 5 min^{-1} (P < 0.01) and the G.M.I. decreased from 5906.07 ± 181.71 to 4105.60 ± 49.35. After PS injection in the NTS, the T.A.C.W. decreased from 468.72 mm 5 min^{-1} ± 6.42 mm 5 min^{-1} to 467.34 mm 5 min^{-1} ± 5.04 mm 5 min^{-1} (P > 0.05), the T.D.C.W. from 236.96 s 5 min^{-1} ± 8.51 s 5 min^{-1} to 232.38 s 5 min^{-1} ± 16.31 s 5 min^{-1} (P > 0.05), and the G.M.I. from 5797.17 ± 141.87 to 5778.08 ± 125.32 (P > 0.05) (Figure 4A-C).

The inhibition rates of the T.A.C.W. in the 4 nmol NaHS, 8 nmol NaHS, and saline groups were 23.83%, 30.69%, and 0.27%, respectively. The inhibition rates of T.D.C.W. in the 4 nmol NaHS, 8 nmol NaHS, and saline groups were 26.21%, 37.43%, and 2.06%, respectively. The inhibition rates of G.M.I. in the 4 nmol NaHS, 8 nmol NaHS, and saline groups were 18.55%, 30.17%, and 0.30%, respectively (Figure 4D). The data indicated that the inhibition rates of T.A.C.W., T.D.C.W., and G.M.I. were significantly higher in the 8 nmol NaHS group compared to the 4 nmol NaHS group. These findings suggest a dose-dependent inhibitory effect of NTS injection of NaHS on gastric motility.

PDTC eliminates the inhibitory effect of NaHS on gastric motility

Injection of PDTC followed by NaHS into the NTS eliminated the inhibitory effect of NaHS on gastric motility (Figure 5A, n = 6). The T.A.C.W. changed from 500.15 mm 5 min^{-1} ± 7.56 mm 5 min^{-1} to 491.06 mm 5 min^{-1} ± 17.19 mm 5 min^{-1} (P > 0.05), the T.D.C.W. changed from 169.84 s 5 min^{-1} ± 3.40 s 5 min^{-1} (P > 0.05), and the G.M.I. changed from 5494.78 ± 140.32 to 5490.60 ± 88.80 after PDTC followed by NaHS injection (P > 0.05) (Figure 5B-D). These data suggest that NaHS can regulate gastric motility through the NF-κB signaling pathway.

Capsazepine eliminates the inhibitory effect of NaHS on gastric motility

Injection of Capsazepine followed by NaHS into the NTS eliminated the inhibitory effect of NaHS on gastric motility (Figure 6A, n = 6). As a result, the T.A.C.W. changed from 514.46 mm 5 min^{-1} ± 6.56 mm 5 min^{-1} to 523.87 mm 5 min^{-1} ± 9.21 mm 5 min^{-1} (P > 0.05), the T.D.C.W. changed from 175.90 s 5 min^{-1} ± 4.22 s 5 min^{-1} to 172.13 s 5 min^{-1} ± 4.68 s 5 min^{-1} (P > 0.05), and the G.M.I. changed from 5932.97 ± 104.93 to 5946.45 ± 184.14 (P > 0.05) (Figure 6B-D). These data suggest that NaHS can regulate gastric motility through TRPV1 channels.

L703606 eliminates the inhibitory effect of NaHS on gastric motility

Injection of L703606 followed by NaHS into the NTS eliminated the inhibitory effect of NaHS on gastric motility (Figure 7A, n = 6). The T.A.C.W. changed from 494.46 mm 5 min^{-1} ± 11.86 mm 5 min^{-1} to 490.53 mm 5 min^{-1} ± 14.00 mm 5 min^{-1} (P > 0.05), the T.D.C.W. changed from 164.10 s 5 min^{-1} ± 5.53 s 5 min^{-1} to 158.39 s 5 min^{-1} ± 10.64 s 5 min^{-1} (P > 0.05), and the G.M.I. changed from 5827.59 ± 133.74 to 5762.80 ± 114.34 (P > 0.05) (Figure 7B-D). These data suggest that NaHS can act on NK1 receptors to regulate gastric motility.

DISCUSSION

Endogenous H_2S concentrations in the brain range between 10 nM and 160 nM[32], and approximately 33% of H_2S is produced by volatilization in NaHS solutions when measurements are made in a closed environment. H_2S (10 mmol/L) in NTS neurons can maintain excitatory postsynaptic potential excitation for 10 minutes, which is equivalent to the time it takes for a microinjection NaHS to work[33]. Moreover, H_2S can cross the cell membrane by free diffusion to modulate cellular properties[34]. We selected NaHS as an exogenous H_2S rapid drug-delivery donor.

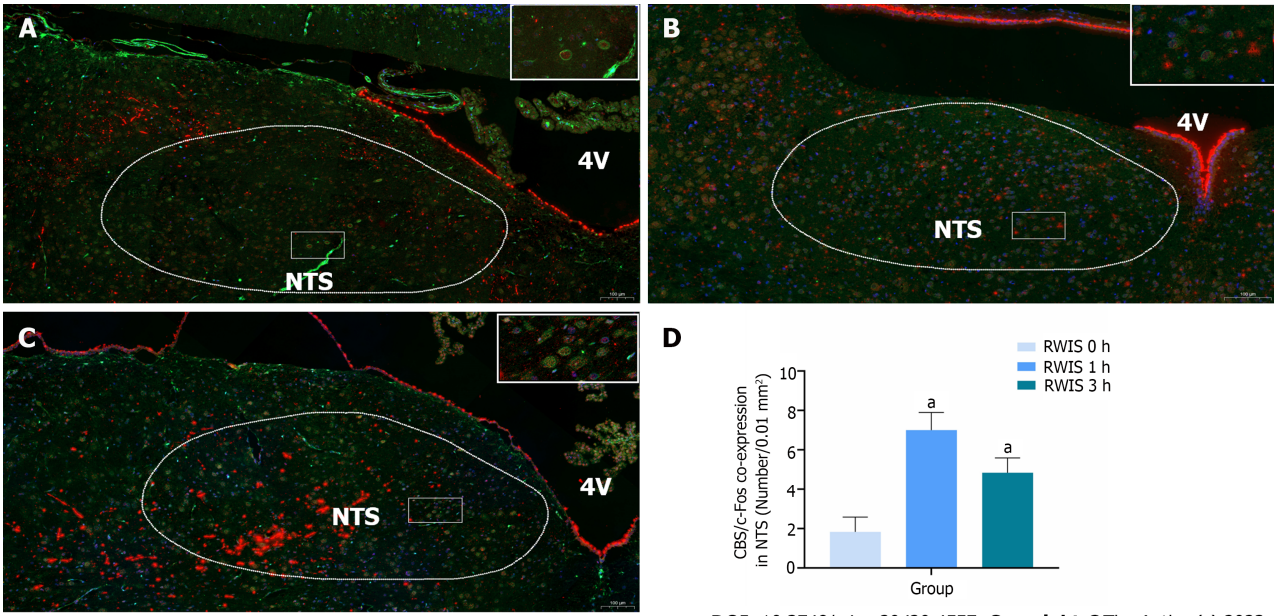

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Figure 1 Co-expression of cystathionine beta-synthase (green) and c-Fos (red) neurons in the nucleus tractus solitarius at different times of restraint water-immersion stress. A: Expression of cystathionine beta-synthase (CBS) and c-Fos in restraint water-immersion stress (RWIS) at 0 h; B: Expressions of CBS and c-Fos in RWIS at 1 h; C: Expression of CBS and c-Fos in RWIS at 3 h; D: Quantification of neurons co-expressing CBS and c-Fos in the nucleus tractus solitarius (n = 6). $^aP < 0.01$ *vs* RWIS 0 h group. NTS: Nucleus tractus solitarius; RWIS: Restraint water-immersion stress; CBS: Cystathionine beta-synthase.

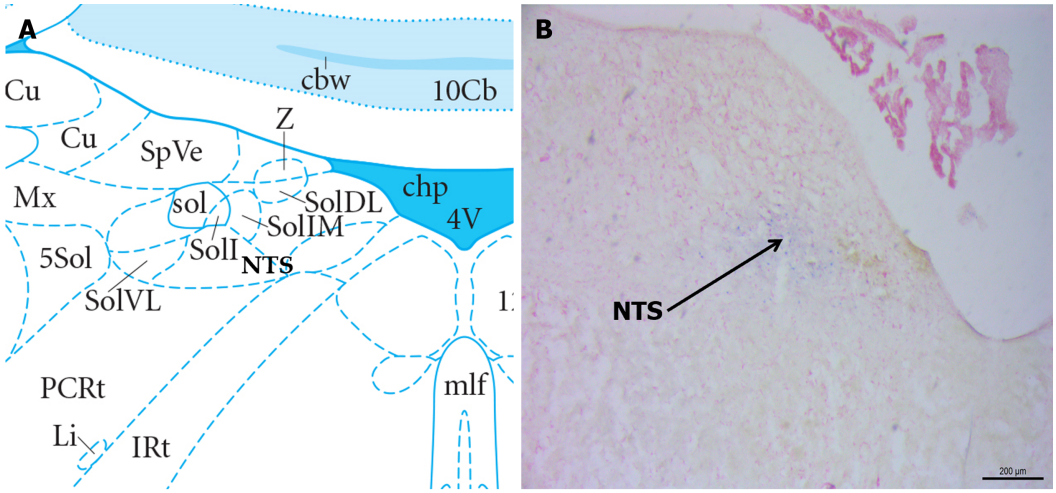

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Figure 2 Histological identification of microinjections located at the nucleus tractus solitarius in the brain. A: Location of the nucleus tractus solitarius (NTS) in the brain atlas; B: Neutral red stained brain section with blue dot sites representing injection into the NTS. NTS: Nucleus tractus solitarius.

H_2S, an emerging gaseous signaling molecule, plays an important role in regulating digestion and the nervous system [35]. Placing coronal NTS slices into NaHS solution was found to cause rapid concentration-dependent depolarization of neurons at NTS sites, with H_2S increasing the postsynaptic currents in NTS neurons by promoting presynaptic glutamate release[36]. Herein, we observed a significant increase in the co-expression of neurons between CBS and c-Fos in rat NTS after RWIS, indicating that H_2S in the NTS is involved in gastrointestinal regulation and stress.

In this experiment, we found that the amplitude and duration of gastric motility and the index of gastric motility were significantly lower in the NTS than in the control group, in a dose-dependent manner, after the injection of different concentrations of NaHS in rats. Sensory information from the upper gastrointestinal tract is transmitted to the NTS *via* vagal afferent fibers, and c-Fos-positive neurons are significantly increased in the NTS following stressful processes. Glutamate release from vagal afferent fibers activates NTS neurons, which can regulate gastrointestinal activity by inhibiting the vagal excitatory cholinergic efferent pathway *via* the inhibitory neurotransmitter GABA or by exciting the vagal inhibitory non-adrenergic non-cholinergic (NANC) efferent pathway[37]. In addition, vagal afferent nerves from the gastrointestinal tract can activate the NANC efferent pathway leading to gastric smooth muscle relaxation. Vagal

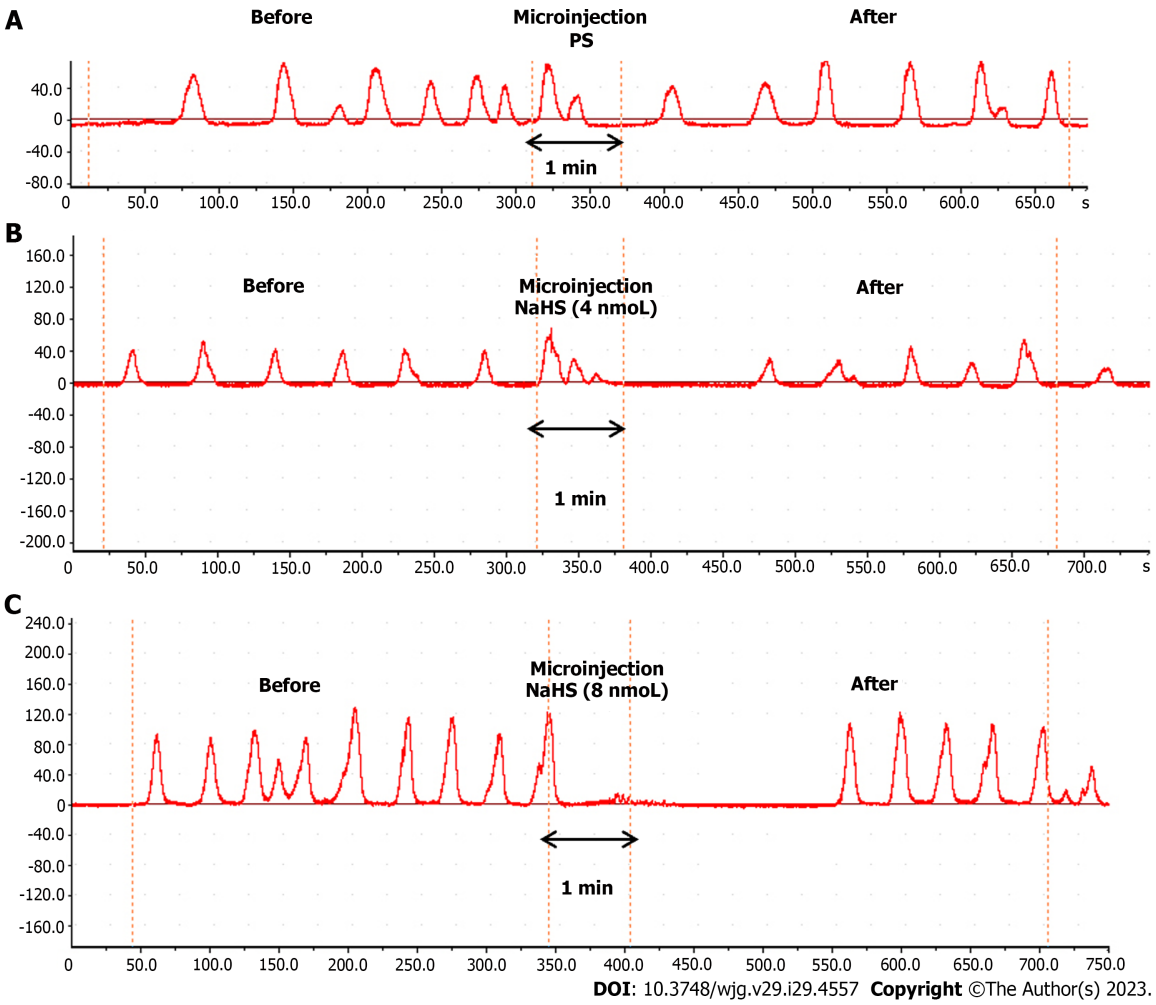

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Figure 3 Effect of microinjection of drugs in the nucleus tractus solitarius on gastric motility in rats. A: Gastric motility curves were recorded in rats microinjected with saline at the nucleus tractus solitarius (NTS); B: Gastric motility curves were recorded in rats microinjected with 4 nmol NaHS in the NTS; C: Gastric motility curves were recorded in rats microinjected with 8 nmol NaHS in the NTS. PS: Physiological saline.

efferent fibers activate the noradrenergic neurons in the NTS, which in turn activate the NANC pathway neurons in the DMV. These NANC-DMV neurons transmit to the gastrointestinal plexus to activate postganglionic cholinergic neurons, thus causing gastric relaxation[38].

In vivo studies found that the microinjection of D-glucose into the NTS resulted in decreased gastric motility and increased intragastric pressure in rats *via* K^+-ATP channel relaxation of the smooth muscles and increased firing of GABAergic neurons[39]. Injection of cholecystokinin in the rat NTS modulates the gastrointestinal motility and secretory function in the upper gastrointestinal tract by activating postganglionic cholinergic excitatory or NANC inhibitory pathways[40]. Increased glutamate content within the NTS directly activates excitatory postsynaptic potentials in NTS neurons, which in turn stimulates local circuit GABAergic and glutamatergic neurons. GABAergic signals at the NTS determine the state of the gastric tone and contraction and mediate changes in gastric mechanical function at the onset of the vagal-vagal reflex[41,42]. The intra-NTS injection of GLP-1 reduces the gastric tone by activating NANC and delays gastric emptying in a dose-dependent manner[43]. Therefore, NaHS injection in the rat NTS may inhibit gastric motility by releasing inhibitory neurotransmitters such as GABA from preganglionic cholinergic neurons.

The activation of TRPV1 leads to the release of various pro-inflammatory cytokines, which can activate NF-kB translocation to the nucleus. Our experiments revealed that the injection of the NF-κB pathway blocker PDTC followed by NaHS eliminated the modulation of gastric function by NaHS. H_2S is mainly synthesized in the brain by the CBS enzyme, andwe found that NaHS injection in both the ambiguous and paraventricular nuclei inhibited gastric motility in rats *via* the NF-κB pathway[44,45], that the injection of NaHS to the brain blocked inflammation-associated apoptosis, and that treatment with NaHS reduced the expression of inflammatory factors in astrocytes and microglia due to Alzheimer's disease[46]. H_2S reduces the levels of phosphorylated p38 MAPK and phosphorylated p65 NF-κB *in vivo*. NaHS also reduces LPS-induced inflammation by inhibiting p38 MAPK and p65 NF-κB in rat cells[47]. SP is the main presynaptically released excitatory transmitter from injured primary afferent fibers. It binds to NK1 receptors on the postsynaptic membrane to activate NF-kB-induced inflammatory factor synthesis. NF-κB is reportedly involved in the transcriptional regulation of various response-related genes, including gastrointestinal mucosal damage, and that the downregulation of NF-κB signaling can inhibit stress-induced local inflammatory responses in the gastric mucosa and can repair local lesions in the gastric mucosa[48,49]. Therefore, physiological concentrations of H_2S can modulate the inflam-

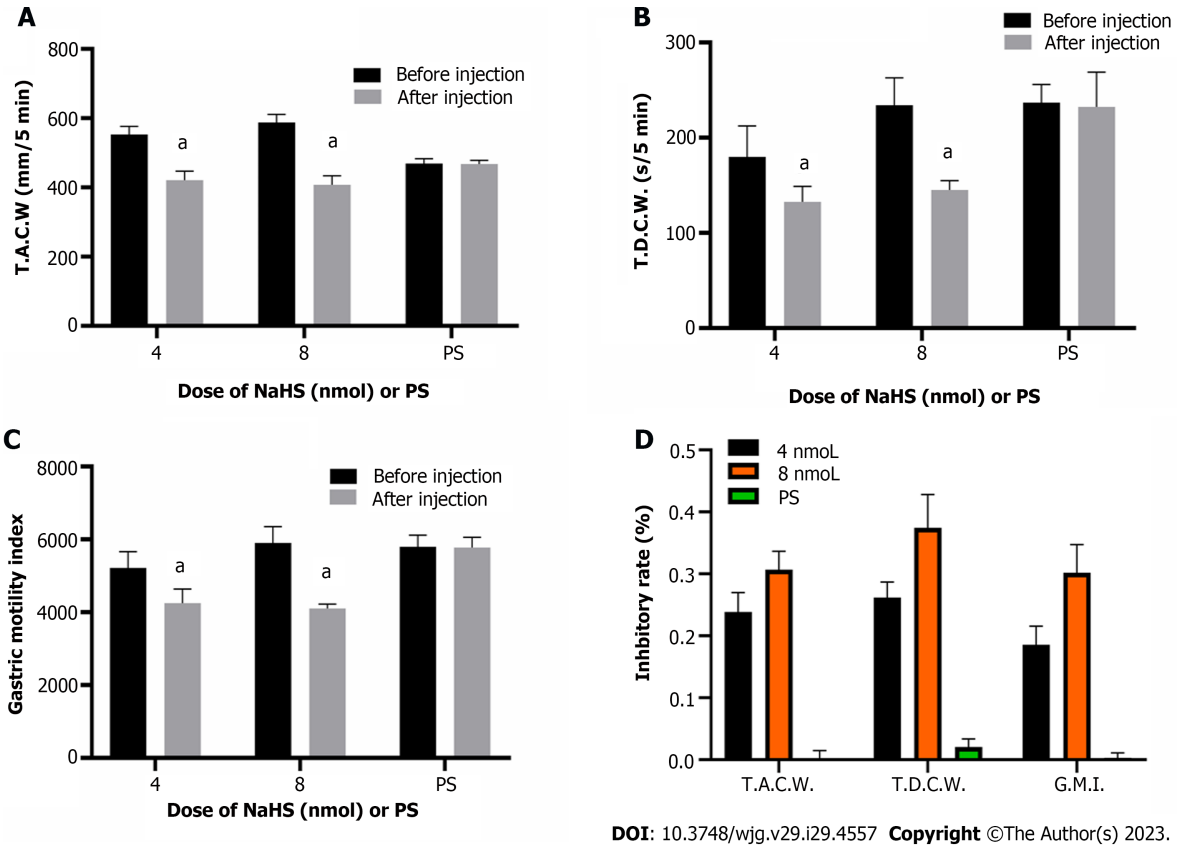

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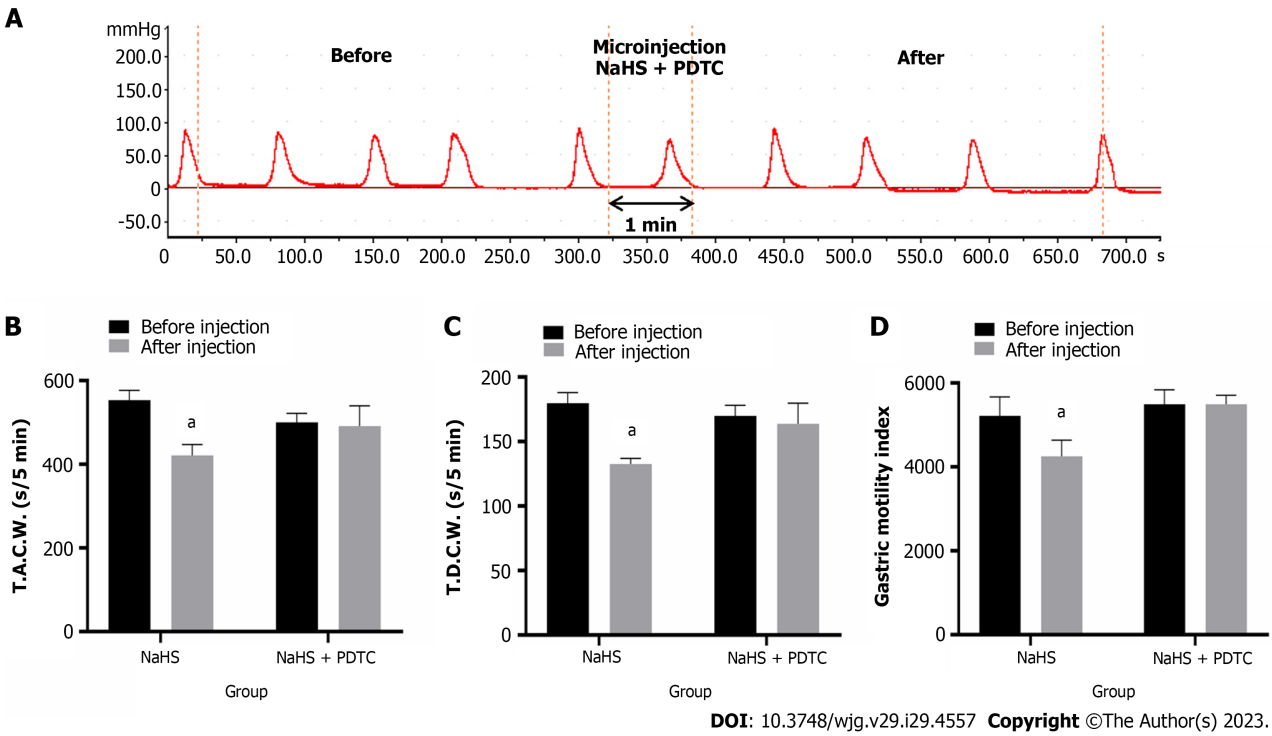
Figure 4 Effects of the microinjection of NaHS (4 nmol and 8 nmol) and physiological saline into the nucleus tractus solitarius before and after the gastric motility data. A: Total amplitude of the contraction wave (T.A.C.W.); B: Total duration of the contraction wave (T.D.C.W.); C: Gastric motility index (G.M.I.); D: Inhibition rates of T.A.C.W., T.D.C.W., and G.M.I. $^aP < 0.01$ *vs* before injection. PS: Physiological saline; T.A.C.W.: Total amplitude of the contraction wave; T.D.C.W.: Total duration of the contraction wave; G.M.I.: Gastric motility index.

matory process and regulate gastric dysfunction by down-regulating the inflammatory response.

To investigate the signaling pathways involved in H_2S regulation, we focused on the TRPV1-SP-NF-kB pathway[50]. TRPV1 activation, enhanced pro-inflammatory cytokine expression, and oxidative stress *via* the NF-kB pathway led to reduced phosphorylation of the ERK signaling pathway, activation of PAG involved in the ERK-NF-kB pathway, and production of SP, which in turn regulated TRPV1-mediated neurogenic inflammation[51]. Our experiments showed that the administration of the TRPV1 blocker Capsazepine followed by NaHS eliminated the modulation of gastric function by NaHS. TRPV1 is widely expressed in spinal and vagal afferent neurons. It innervates the gastrointestinal tract, and its upregulated sensitivity may be associated with the pathophysiological functions of diseases such as visceral pain, irritable bowel syndrome, inflammatory bowel disease and pancreatitis[52]. TRPV1 presents two sides to inflammation. There are even studies showing the alternating effects of TRPV1 on inflammation[53]. Capsaicin induces inflammation in the stomach by activating TRPV1, damages the gastrointestinal mucosa, causes structural changes in the intestinal barrier and further leads to other gastrointestinal symptoms[54-56]. It also decreases the expression of anti-inflammatory cytokines in the stomach and intestine and promotes the release of gastrointestinal neuropeptide SP, which is closely associated with gastrointestinal visceral pain[57]. The findings suggest that the TRPV1 receptor antagonist caspofungin abrogates H_2S donor-induced enhancement of gastric emptying and that TRPV1-dependent pathways have been shown to produce modulation of vagally mediated muscle contractions in the gastrointestinal tract[58]. Capsaicin also activates the enterocholinergic neurons in guinea pig's small intestine and induces contractile effects and inhibition of gastric emptying, thus inducing the relaxation of smooth muscles of the fundus[59]. Capsazepine was also found to inhibit NaHS-induced pyloric smooth muscle relaxation, indicating that the effect of H_2S donors on enhancing gastric emptying and inducing pyloric sphincter relaxation is mediated by the activation of TRPV1 receptors. Although there are many mechanisms by which this effect can be manifested, our data support the theory of smooth muscle relaxation induced by afferent neuronal TRPV1 receptor activation[45,60].

TRPV1 receptors may act as molecular sensors involved in processing cardiac injury information in spinal neurons. Harmful environmental stimuli can activate TRPV1 to produce pro-inflammatory mediators in the epithelial cells, thus triggering neurogenic inflammatory responses. TRPV1 antagonists reportedly inhibited H_2S-induced neuropeptide release and bronchoconstriction in vitro, whereas H_2S induced the release of an endogenous tachykinin, SP, by stimulating sensory nerve endings, and that NaHS-induced SP release is significantly reduced when the TRPV1 blocker Capsazepine is applied[61]. Capsaicin also slows down gastric motility through TRPV1-induced excitation of gastric sensory nerve fibers[62]. A possible role of capsaicin-sensitive vagal afferent nerves in gastric mucosal injury and prevention has been demonstrated and is associated with the release of SP[31].



DOI: 10.3748/wjg.v29.i29.4557

Figure 5 Effect of microinjection of 4 nmol NaHS and 4 nmol NaHS + pyrrolidine dithiocarbamate into the nucleus tractus solitarius on gastric motility in rats. A: Gastric motility curve recorded in rats with 4 nmol NaHS + pyrrolidine dithiocarbamate microinjection in nucleus tractus solitarius; B: Data of total amplitude of the contraction wave; C: Data of total duration of the contraction wave; D: Data of gastric motility index. $^aP < 0.01$ *vs* before injection. PDTC: Pyrrolidine dithiocarbamate; T.A.C.W.: Total amplitude of the contraction wave; T.D.C.W.: Total duration of the contraction wave.

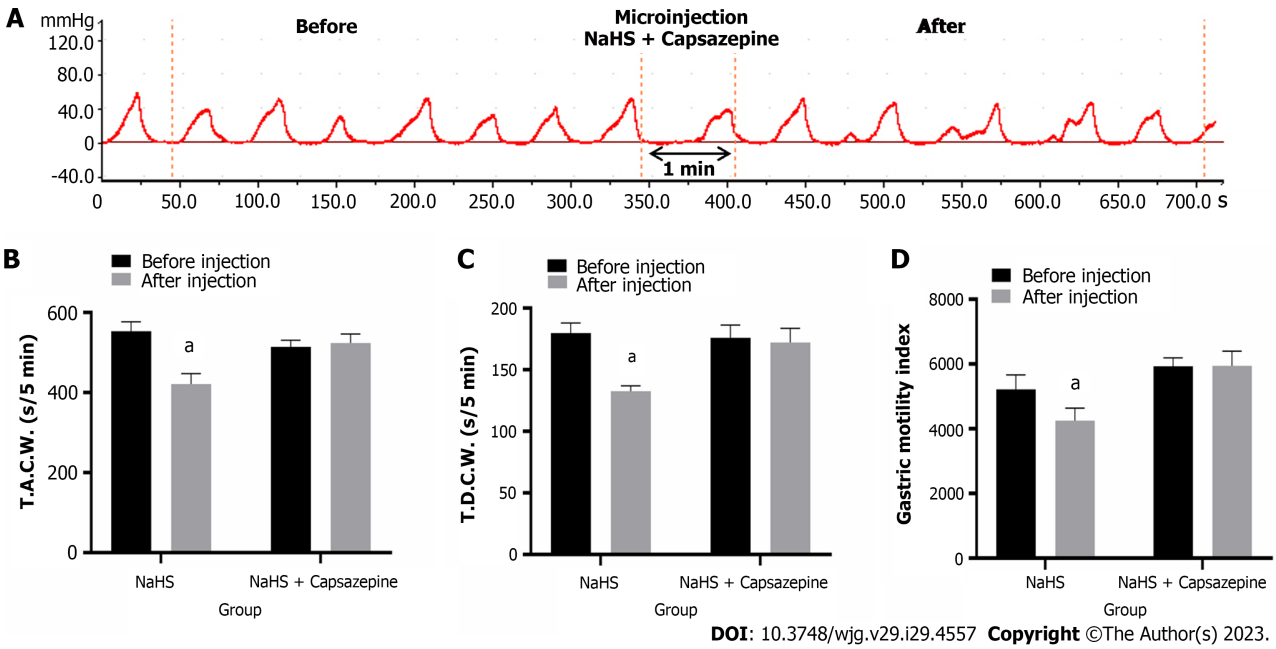


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Figure 6 Effects of microinjection of 4 nmol NaHS and 4 nmol NaHS + Capsazepine into the nucleus tractus solitarius on gastric motility in rats. A: Gastric motility curve recorded in rats with 4 nmol NaHS + Capsazepine microinjection in nucleus tractus solitarius; B: Data of total amplitude of the contraction wave; C: Data of total duration of the contraction wave; D: Data of gastric motility index. $^aP < 0.01$ *vs* before injection. T.A.C.W.: Total amplitude of the contraction wave; T.D.C.W.: Total duration of the contraction wave.

We eliminated the inhibitory effect of NaHS on gastric motility in our experiments by injecting the NK1 receptor blocker L703606 followed by NaHS. SP is a brain-gut peptide abundant in mammals that acts as a neurotransmitter for specific receptors to mediate NANC expression in the autonomic nervous system. Numerous studies have shown its role in stress and nociceptive transmission[63]. Injection of exogenous SP into the NTS resulted in the reduction of gastric motility. In contrast, injection of NK1 blockers enhanced the gastric motility. Therefore, SP in the NTS may play a

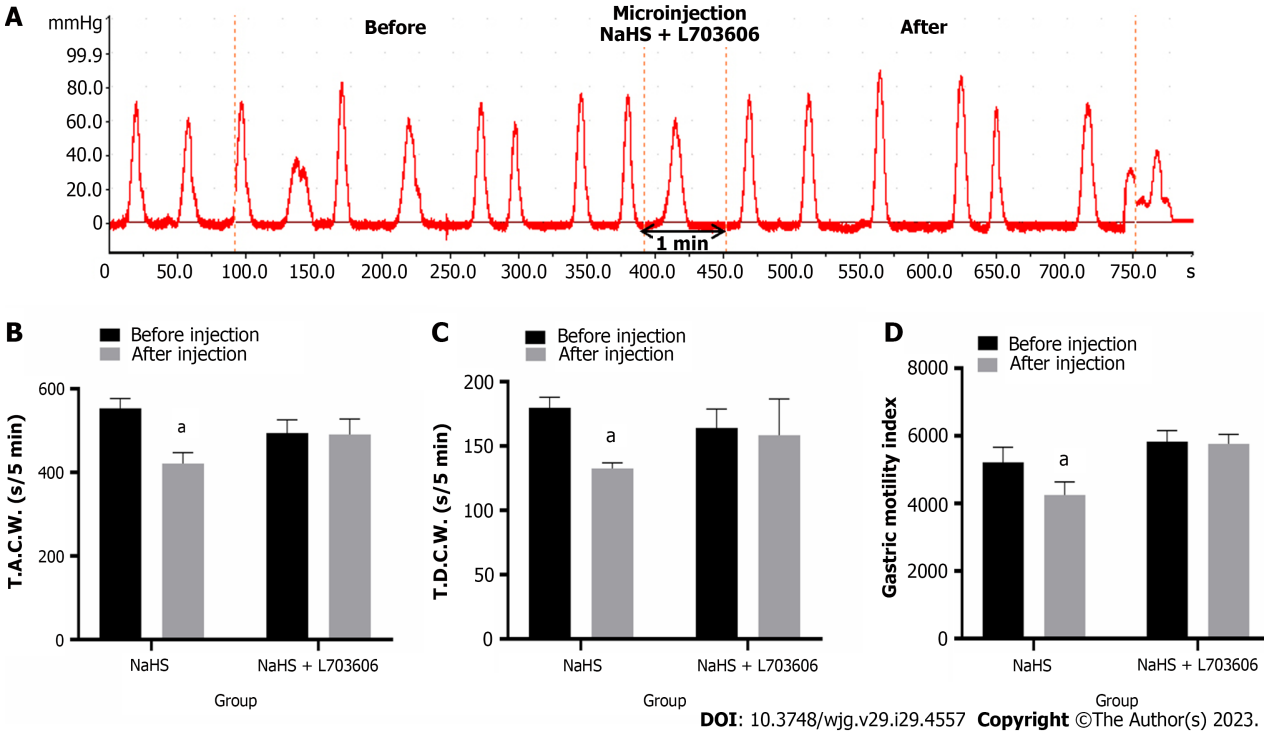

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Figure 7 Effects of microinjection of 4 nmol NaHS and 4 nmol NaHS + L703606 into the nucleus tractus solitarius on gastric motility in rats. A: Gastric motility curve recorded in rats with 4 nmol NaHS + L703606 microinjection in nucleus tractus solitarius; B: Data of total amplitude of the contraction wave; C: Data of total duration of the contraction wave; D: Data of gastric motility index. $^aP < 0.01$ *vs* before injection. T.A.C.W.: Total amplitude of the contraction wave; T.D.C.W.: Total duration of the contraction wave.

predominantly inhibitory role in gastrointestinal regulation. Administration of NK1 receptor antagonists also prevented H_2S-induced contractile responses, indicating that SP released from sensory nerve endings is the ultimate mediator of H_2S-induced excitation of smooth muscle in rats, possibly leading to altered gastric motility and the formation of gastric ulcers[64]. Injection of glutamate and SP in the NTS modulates gastric motility and emptying, and leads to a dose-dependent decrease in tonic gastric pressure and inhibition of gastric motility[65,66]. It has been shown that glutamate, acting through N-methyl-D-aspartic acid receptors with glutamate ion channels, and tachykinin, acting through NK1 and NK2 receptors, act synergistically in the transmission of acid stimuli from the gastric mucosa to the NTS[67]. SP regulates gastric smooth muscle contraction by both inhibition and enhancement mechanisms. Studies have found that SP microinjections in the brain have inhibitory effects on gastric motility. Furthermore, microinjections of SP in the rat DMV inhibited gastric EMG fast waves and gastric motility. These effects could be abolished by SP receptor antagonists and by severing the vagus nerve, respectively[68]. SP in the brain plays a role in stress-induced physiological and behavioral activity. The effects of NK1 can be blocked using injectable drugs or knockout methods[69-71]. Moreover, c-Fos expression can be downregulated, with the nucleus involved in stress in the brain and pain, causing pain through increased SP release in the hypothalamic and spinal cord tissue. This results in a sterile inflammatory response, with persistent pain leading to increased levels of SP receptors in the posterior horn of the spinal cord[72]. SP also plays a role in transmitting injurious information, with low doses given ventrally to produce analgesia, acting in a neuroendocrine manner on various immune cells involved in immune regulation and enhancing immune function[73]. We, therefore, consider that H_2S enhances the inhibitory effect of SP on gastric motility by excitation of NTS neurons which are then transmitted to the DMV.

CONCLUSION

Our experiments revealed that CBSergic neurons affecting gastric function were present in the NTS and were activated by stress. Furthermore, exogenous H_2S in the NTS significantly inhibited gastric motility in rats, possibly by activating the TRPV1 pathway through NF-κB channels to release SP. This is the first study to report that H_2S in the NTS may regulate gastric function. It provides an important experimental basis for the clinical prevention and treatment of gastric ulcers.

ARTICLE HIGHLIGHTS

Research background

Recent studies have revealed that hydrogen sulfide is the third class of gas signaling molecules after nitric oxide (NO) and carbon monoxide (CO). The high level of endogenous hydrogen sulfide found in the brain, which is mainly produced by cystathionine beta-synthase, suggests that it may have a physiological function, and the nucleus tractus solitarius is important nucleus that regulates the function of internal organs, so we want to elucidate the role of hydrogen sulfide in the NTS in regulating gastric function in rats.

Research motivation

To investigate whether hydrogen sulfide in the nucleus tractus solitarius is involved in the regulation of gastric dysfunction by restraint water-immersion stress, this study will examine the role of hydrogen sulfide in the nucleus tractus solitarius in the regulation of gastric motility.

Research objectives

It is the first time to propose that hydrogen sulfide in the nucleus tractus solitarius has a regulatory effect on the gastric motility caused by restraint water-immersion stress, and to investigate its mechanism of action, which can not only elucidate the mechanism of regulation of gastric dysfunction by hydrogen sulfide in the nucleus tractus solitarius, and also provide an important experimental basis for the prevention and treatment of stress gastric ulcer from the central aspect in clinical practice.

Research methods

We used immunohistochemical, fluorescent double-labeling technique and restraint water-immersion stress model to confirm the involvement of hydrogen sulfide-producing cystathionine beta-synthase neurons in the nucleus tractus solitarius in the regulation of gastric function, and physiological methods to record changes in gastric motility before and after their brain injection.

Research results

After restraint water-immersion stress, cystathionine beta-synthase neurons containing c-Fos were significantly increased and gastric motility was inhibited in rats after nucleus tractus solitarius injection of NaHS, and this inhibitory effect was eliminated after pre-injection of transient receptor potential vanilloid 1 channels, NF-κB channel blockers, and NK1 receptor antagonists followed by NaHS injection.

Research conclusions

Injection NaHS into the nucleus tractus solitarius can inhibit gastric motility in rats and this effect may be mediated by TRPV1 and NK1 receptors *via* NF-κB channel-dependent activation.

Research perspectives

Our next step will be to continue our work on the effects of endogenous hydrogen sulfide in the nucleus tractus solitarius on gastric function.

FOOTNOTES

Author contributions: Sun HZ and Li CY contributed equally to this work; Sun HZ conceived and designed the experiments; Li CY, Shi Y, Li JJ, Wang YY, Han LN, Zhu LJ, and Zhang YF performed the experiments; Sun HZ and Li CY analyzed the data; Sun HZ provided reagents/materials/analysis tools; Li CY authored the paper; all authors have read and approved the final manuscript.

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ORIGINAL ARTICLE

Case Control Study

Comparison of modified gunsight suture technique and traditional interrupted suture in enterostomy closure

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Abstract

BACKGROUND

Prophylactic enterostomy surgery is a common surgical approach used to reduce the risk of anastomotic leakage in patients who have undergone partial intestinal resection due to trauma or tumors. However, the traditional interrupted suturing technique used in enterostomy closure surgery has several issues, including longer surgical incisions and higher incision tension, which can increase the risk of postoperative complications. To address these issues, scholars have proposed the use of a "gunsight suture" technique. This technique involves using a gunsight incision instead of a traditional linear incision, leaving a gap in the center for the drainage of blood and fluid to reduce the risk of infection. Building on this technique, we propose an improved gunsight suture technique. A drainage tube is placed at the lowest point of the incision and close the gap in the center of the gunsight suture, which theoretically facilitates early postoperative mobility and reduces the burden of dressing changes, thereby reducing the risk of postoperative complications.

AIM

To compare the effectiveness of improved gunsight suture technique with traditional interrupted suture in closing intestinal stomas.

METHODS

In this study, a retrospective, single-center case analysis was conducted on 270

patients who underwent prophylactic ileostomy closure surgery at the Department of Colorectal Surgery of Qilu Hospital from April 2017 to December 2021. The patients were divided into two groups: 135 patients received sutures using the improved gunsight method, while the remaining 135 patients were sutured with the traditional interrupted suture method. We collected data on a variety of parameters, such as operation time, postoperative pain score, body temperature, length of hospital stays, laboratory indicators, incidence of incisional complications, number of wound dressing changes, and hospitalization costs. Non-parametric tests and chi-square tests were utilized for data analysis.

RESULTS

There were no statistically significant differences in general patient information between the two groups, including the interval between the first surgery and the stoma closure [132 (105, 184) d *vs* 134 (109, 181) d, $P = 0.63$], gender ratio (0.64 *vs* 0.69, $P = 0.44$), age [62 (52, 68) years *vs* 60 (52, 68) years, $P = 0.33$], preoperative body mass index (BMI) [23.83 (21.60, 25.95) kg/m² *vs* 23.12 (20.94, 25.06) kg/m², $P = 0.17$]. The incidence of incision infection in the improved gunsight suture group tended to be lower than that in the traditional interrupted suture group [($n = 2/135$, 1.4%) *vs* ($n = 10/135$, 7.4%), $P < 0.05$], and the postoperative hospital stay in the improved gunsight suture group was significantly shorter than that in the traditional interrupted suture group [5 (4, 7) d *vs* 7 (6, 8) d, $P < 0.05$]. Additionally, the surgical cost in the modified gunsight suture group was slightly lower than that in the traditional suture group [4840 (4330, 5138) yuan *vs* 4980 (4726, 5221) yuan, $P > 0.05$], but there was no significant difference in the total hospitalization cost between the two groups.

CONCLUSION

In stoma closure surgery, the improved gunsight technique can reduce the incision infection rate, shorten the postoperative hospital stay, reduce wound tension, and provide better wound cosmetic effects compared to traditional interrupted suture.

Key Words: Enterostomy; Abdominal wound closure technique; Suture techniques; Surgical wound infection; Hospital costs; Hospital stay

Core Tip: Based on the gunsight technique, we proposed an improved gunsight closure method for enterostomy surgery in this study. This closure method can reduce the incision tension, lower the risk of incision infection, decrease the length of hospital stay, and reduce hospitalization costs. Additionally, it requires less postoperative wound care and provides a better cosmetic outcome.

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INTRODUCTION

Patients with colon cancer, inflammatory bowel disease, or intestinal trauma may require partial bowel resection. However, a simple one-stage surgery may result in complications such as anastomotic leakage, significantly affecting patient health and increasing the economic burden. To reduce this risk, a prophylactic intestinal stoma is often performed in patients at high risk for anastomotic leakage during routine surgery[1,2]. After 2-3 mo, the patient's condition was evaluated and a second-stage anastomosis was performed to restore gastrointestinal continuity[3]. Although prophylactic intestinal stoma can reduce complications such as anastomotic leakage[4], second-stage anastomotic surgery also has its own risks, including incision infection, intestinal obstruction, and incisional hernia[5-8]. Unlike Class II incisions in other gastrointestinal surgeries, the skin around the colostomy site is continuously exposed to feces, classifying it as a Class III incision. Incision infections are particularly common, with an incidence of up to 41% reported[9]. Due to factors, such as contamination of the intestinal contents, preventing incision infections may be challenging.

According to guidelines published by different countries, surgical site infection increases hospital stay and the risk of death by 2-11 times[10-13]. To address this issue, researchers have proposed improved surgical methods, such as placing drainage tubes at the incision site, selecting special suture materials, and modifying the suture technique[14-19]. Some special antimicrobial suture materials and techniques are highly regarded; however, there is currently no recognized surgical procedure to reduce the incidence of incision infections. Studies have shown that compared with traditional interrupted sutures, the purse-string suture technique can effectively drain subcutaneous fluid and significantly reduce the incidence of incision infections. However, owing to the high tension at the center of the incision[20-22], the healing

process of the abdominal incision after closure may take up to 3 wk.

In 2010, Lim *et al*[23] proposed a suture technique called "gunsight suture" that can reduce tension at the incision site and leave a smaller drainage gap at the center of the incision. This technique can effectively drain subcutaneous blood and fluid, reduce infection rate, and provide good cosmetic results with minimal scarring[20,24,25]. However, it is difficult to drain all the subcutaneous blood and fluid by placing a drainage tube or strip at an angle in the central gap. In addition, the vertical placement of the drainage tube significantly affects the postoperative activity range, and frequent dressing changes are required owing to leakage at the center of the incision, which can cause serious psychological and economic burdens on patients. Moreover, fixing the drainage tube in the central gap of the gunsight suture incision is challenging; therefore, drainage strips are often used, resulting in a shorter drainage time and a slightly inferior drainage effect.

Therefore, in our hospital, we reserve a smaller gap (approximately 0.2 cm) at the center and place a drainage tube through the entire subcutaneous gap in a small incision next to the incision. The catheter was left in place for approximately 14 d and removed after the incision healed (Figure 1). This design effectively drained the subcutaneous fluid and reduced the local tension at the incision site (Figure 1C). Theoretically, it can reduce the incidence of incision infection, shorten the postoperative hospital stay, provide better cosmetic results, and reduce hospitalization costs. To verify the significance of the improved gunsight suture method in clinical practice, data were collected from 270 patients who underwent ileostomy closure surgery between April 2017 and December 2022. Our findings are reported below.

MATERIALS AND METHODS

Study design

This retrospective case-control study was conducted between April 2017 and December 2022 to evaluate the effectiveness of a modified surgical technique for ileostomy closure and lateral ileal anastomosis. This study was approved by the Ethics Committee of Qilu Hospital, Shandong University (Approval No: KYLL-2020-120).

Participants

A total of 270 patients who underwent ileostomy closure and ileo-ileal lateral anastomosis surgery at the Department of Colorectal Surgery at Qilu Hospital were included in this study.

Inclusion criteria: Age > 18 years, patients who underwent ileostomy closure and ileo-ileal lateral anastomosis surgery, no stenosis of the intestinal lumen near the stoma on preoperative colonoscopy, and no concomitant colitis.

Exclusion criteria: Patients who required hernia repair due to concomitant incisional hernia, patients with mental dysfunction, and patients with other tumors, serious illnesses, or severe abdominal adhesions.

Preoperative preparation

The day before surgery, the patient underwent mechanical bowel preparation with polyethylene glycol and rectal cleansing enema. This procedure improved the patient's general condition, eliminated distant obstruction, and controlled the inflammation around the stoma. Prophylactic antibiotics were administered 30 min before surgery to prevent infection.

Surgical procedure

After the patients were fully anesthetized, they were placed in the supine position. First, a 2-0 silk suture was used to close the ileostomy and prevent intestinal contents from spilling out during the reduction process. After re-disinfecting, an appropriate stoma incision was made using the suturing method. The tissues surrounding the stoma were separated layer by layer to avoid damage to the intestinal wall. The freed intestine was then separated in all directions, and the direction with less adhesion was chosen to cut open the peritoneum and enter the abdominal cavity where the adhesions were loosened. The two ends of the freed intestinal tube were pulled out of the abdominal wall by > 5 cm, and a linear cutting closure device was used to perform a side-to-side anastomosis of the proximal and distal ends of the ileum to restore intestinal continuity. The 4-0 absorbable suture (Vycrl, Ethicon) was used to intermittently suture and reinforce the ileal muscularis layers. The bowel is returned to the abdominal cavity without placement of an abdominal drainage tube. Subsequently, a continuous suture is performed using 2-0 absorbable antibacterial sutures (VCP-603) to close the peritoneum and rectal sheath, leaving a subcutaneous drainage tube in place. The appropriate skin closure method is chosen based on the group.

Simple interrupted suture group (A, B): No or only minimal trimming is required at the site of repositioning. A 2-0 suture is used for full-thickness vertical mattress suturing, with a needle margin of 1 cm, a stitch spacing of 2 cm, and avoiding residual ineffective cavities. Reinforcement sutures can be placed between every two stitches, with a needle margin of 0.5 cm. This approach can better support skin closure and reduce tension on the surgical incision. After disinfection, sterile auxiliary materials are used for compression and bandaging.

Modified gunsight suture group (C, D): The incision was marked at the 3, 6, 9, and 12 o'clock positions, and the skin tissue between the marked points was triangularly excised to form a gunsight incision. A 2-0 silk suture was used to suture the subcutaneous tissue at the four marked points, and the skin was tightened and knotted after closure. The central part of the incision was brought together as closely as possible without leaving or leaving a gap of approximately

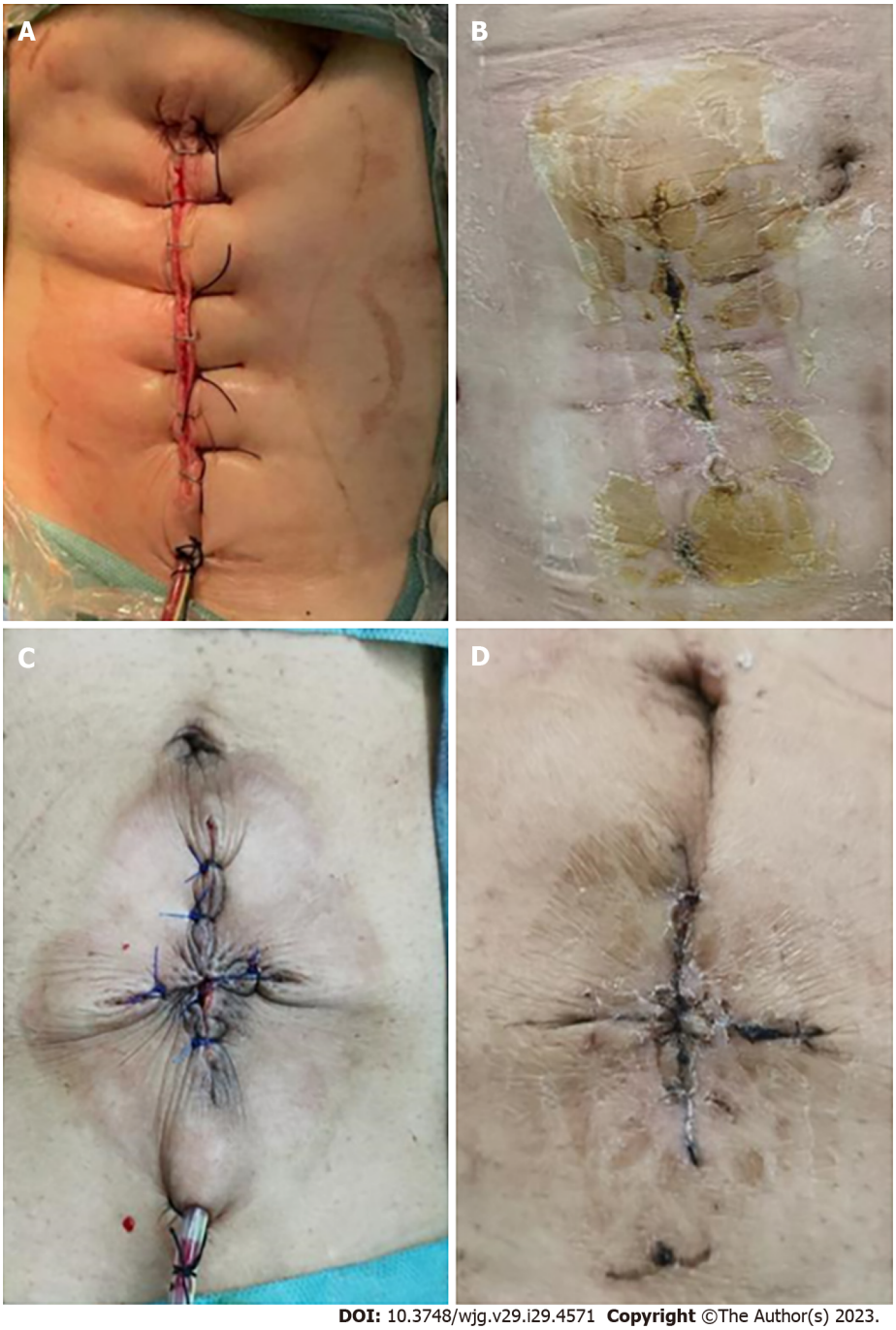

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Figure 1 Surgical stitching result diagram. A: Photo of incision closed with traditional interrupted suture; B: Postoperative photo of incision healing after traditional interrupted suture; C: Photo of incision closed with improved gunsight suture; D: Postoperative photo of incision healing after improved gunsight suture.

0.2 cm. Finally, a 2-0 silk suture was used to suture the midpoints of the four edges of the gunsight incision to complete the reinforcement. After disinfection, sterile auxiliary materials were applied for compression and bandaging.

Observation indicators

The patients' baseline data included age, sex, surgery time, postoperative pain score, postoperative temperature changes, hospitalization time, postoperative laboratory indicators, incision infection rate, and hospitalization costs. The temperature was recorded for 3 d after surgery. The Numeric Rating Scale (NRS) was used to assess the pain scores.

Statistical analysis

Data analysis was performed using the SPSS 26 statistical software. Normally distributed metric data were expressed as mean ± SD and intergroup comparisons were performed using independent sample *t*-tests. Non-normally distributed quantitative data were expressed as median (interquartile range) [M (IQR)], and intergroup comparisons were performed using the Mann-Whitney *U* test. Categorical variables were expressed as frequencies, and intergroup comparisons were

performed using Chi-squared test or Fisher's exact test. Statistical significance was set at $P < 0.05$.

RESULTS

The study included 270 patients who underwent stoma closure surgery at Qilu Hospital. All the patients completed the procedure without anastomotic leakage. Among them, 135 patients received modified Gunsight sutures, and 135 received traditional simple interrupted sutures. Statistical comparisons showed no significant differences in sex ratio, age, preoperative BMI, or the proportion of patients with diabetes between the two groups (Table 1).

Laboratory indicators included the systemic immune inflammation index (SII), red blood cell count, and hemoglobin levels. The SII was calculated as the platelet count multiplied by the ratio of neutrophils to lymphocytes and was used as a simple and effective evaluation index to assess the immune and inflammatory status and prognosis of patients before and after colorectal surgery. Previous studies have shown that the SII is an independent risk factor for the prognosis of colorectal cancer patients. There were no statistically significant differences in the laboratory indicators between the two groups[26-28].

According to the comparison of the main observation indicators (Table 2), the incidence of surgical site infection (SSI) in the modified gunsight suture group was lower than that in the traditional simple interrupted suture group [(n = 2/135, 1.4%) *vs* (n = 10/135, 7.4%), $P < 0.05$], and the length of hospital stay and the number of dressing changes were also significantly better than those in the traditional simple interrupted suture group. However, there were no significant differences in the postoperative body temperature, pain score, or postoperative first bowel gas passage time between the two groups ($P > 0.05$). In addition, we compared the surgical and hospitalization costs between the two groups and found that the surgical cost in the modified Gunsight suture group was slightly lower than that in the traditional suture group [4840 (4330, 5138) yuan *vs* 4980 (4726, 5221) yuan, $P > 0.05$], but there was no significant difference in the total hospitalization cost between the two groups. Furthermore, scar formation after incision healing was significantly lower in the modified Gunsight suture group than in the control group (Figure 1B and D).

DISCUSSION

During stoma closure surgery in patients who undergo preventive ostomy, there is a high incidence of incisional infections due to environmental contamination, subcutaneous fluid accumulation, and other reasons, with reported rates ranging from 3% to 41%[29]. This delay in wound healing can severely affect patients' quality of life. To reduce the risk of postoperative incisional infections, many clinicians have proposed improvements in the suture method of the incision [20], among which the modified Gunsight suture technique has shown promise.

Reid *et al*[30] conducted a randomized controlled clinical trial involving 61 patients to compare postoperative complications of the purse-string suture technique with those of the traditional simple interrupted suture technique. They found that purse-string sutures had fewer surgical site infections than conventional sutures, with two out of 30 patients experiencing infections compared to 12 out of 31 patients in the conventional suture group[30]. In a multicenter prospective study by Han *et al*[20], the effectiveness of the gunsight and purse-string suture techniques in closing skin incisions was compared. This study found that the gunsight suture technique has a lower incision infection rate, similar to that of the purse-string suture technique. Additionally, patients in the Gunsight suture group had shorter incision healing times and higher patient satisfaction[20]. In a case-review study of 233 patients, Li *et al*[31] compared the clinical characteristics between patients with traditional simple interrupted sutures and those with gunsight sutures. The incidence of surgical site infections was significantly lower in the Gunsight suture group than in the traditional simple interrupted suture group. Gunsight sutures were identified as an independent protective risk factor for surgical site infections, with a dominance ratio of 0.212 and a P value of 0.048.

This study showed that our modified Gunsight suture technique is associated with a lower incidence of surgical site infection when compared with the traditional simple interrupted suture technique. Specifically, 10 patients in the traditional simple interrupted suture group experienced postoperative incisional infections, with three of these patients requiring a second surgical intervention and wound resuturing after failed conservative treatment. In contrast, only two patients in the modified gunsight suture group developed postoperative incisional infections, which resolved after conservative treatment. These findings provide evidence supporting the effectiveness of the modified Gunsight suture technique in reducing the risk of incisional infection.

In addition to the reduced incidence of SSI, patients in the modified Gunsight suture group also experienced shorter postoperative hospitalization days than those in the simple interrupted suture group. This difference in hospitalization time may be due to an increased risk of incision infection and the need for more frequent and longer wound care, such as wet alcohol dressing, in the simple interrupted suture group.

Furthermore, the surgical costs for patients in the gunsight suture group were slightly lower than those in the traditional suture group, and there was no significant difference in total hospitalization costs between the two groups. Furthermore, there were no significant differences between the two groups in terms of postoperative pain scores, exhaust time, and related laboratory indicators. Postoperative pain scores were evaluated using the NRS, with most patients scoring between 1 and 3 indicating mild pain. This may explain the lack of significant differences between the two groups. Additionally, the postoperative exhaust time and laboratory test results were not significantly associated with wound closure.

Table 1 General information

Group	Gunsight suture	Traditional suture	Statistical quantity	*P* value
Case number	135	135	-	-
Male (%)	86 (63.7)	92 (68.1)	$\chi^2 = 0.59$	0.440
Age in yr (IQR)	62 (52, 68)	60 (52, 68)	$Z = -0.97$	0.332
BMI (IQR)	23.83 (21.60, 25.95)	23.12 (20.94, 25.06)	$Z = -1.377$	0.169
DM (%)	17 (12.6)	23 (17.0)	$\chi^2 = 1.06$	0.300
Length of time between two surgeries, d (IQR)	132 (105, 184)	134 (109, 181)	$Z = -0.489$	0.625

BMI: Body mass index [a commonly used index to assess the relationship between weight and height, usually expressed in units of kilograms per square meter (kg/m²)]; DM: Diabetes mellitus (a chronic metabolic disease characterized by prolonged high blood sugar levels); IQR: Interquartile range [a statistical measure used to describe the spread of a dataset, it represents the span of the middle 50% of the data and is calculated as the difference between the first quartile (Q1) and the third quartile (Q3)].

Table 2 Patient observation indicators

Group		Gunsight suture (IQR)	Traditional suture (IQR)	Statistical quantity	*P* value
SII (postoperative/preoperative)		3.24 (2.16, 4.78)	3.14 (2.10, 5.47)	$Z = -0.592$	0.554
Red blood cell (postoperative/preoperative)		0.90 (0.84, 0.95)	0.91 (0.87, 9.96)	$Z = -1.445$	0.148
Haemoglobin (postoperative/postoperative)		0.91 (0.85, 0.97)	0.92 (0.88, 0.96)	$Z = -1.231$	0.218
SSI (%)		2 (1.4)	10 (7.4)	$\chi^2 = -5.580$	0.018
Postoperative intestinal exhaust time (d)		3 (2, 3)	3 (2, 3)	$Z = -1.171$	0.242
Total cost of hospitalization (Yuan)		33569 (30545, 36757)	32782 (29134, 36199)	$Z = -1.199$	0.230
Cost of surgery (Yuan)		4840 (4330, 5138)	4980 (4726, 5221)	$Z = -2.745$	0.006
Number of dressing changes for surgical incision		2 (1, 3)	4 (3, 6)	$Z = -2.460$	< 0.001
Operation time		80 (70, 90)	80 (70, 85)	$Z = -1.210$	0.220
Early postoperative pain score (NRS)	DAY1	3 (3, 3)	3 (3, 3)	$Z = -1.145$	0.252
	DAY2	2 (2, 2)	2 (2, 2)	$Z = -0.044$	0.965
	DAY3	2.0 (1.5, 2.0)	1.5 (1.5, 2.0)	$Z = -0.603$	0.547
Early postoperative body temperature (°C)	DAY1	36.7 (36.4, 36.9)	36.7 (36.4, 36.9)	$Z = -0.476$	0.634
	DAY2	37.4 (37.1, 37.9)	37.6 (37.1, 38.1)	$Z = -1.769$	0.077
	DAY3	37.1 (36.9, 37.4)	37.20 (36.85, 37.50)	$Z = -1.385$	0.166
	DAY4	36.9 (36.7, 37.1)	36.9 (36.7, 37.3)	$Z = -1.315$	0.189
Postoperative hospital stay (d)		5 (4, 7)	7 (6, 8)	$Z = -5.181$	< 0.001

SII: Systemic immune inflammation index (a composite index based on peripheral blood neutrophil, lymphocyte, and platelet counts, used to reflect the systemic inflammatory response in patients with various diseases); SSI: Surgical site infection (an infection that occurs after surgery in the part of the body where the surgery took place); Postoperative Pain Score (NRS): Numeric Rating Scale (a commonly used tool to assess the intensity of postoperative pain. It consists of a scale from 0 to 10, where 0 represents no pain and 10 represents the worst possible pain).

CONCLUSION

The modified Gunsight suture technique is an effective method for reducing the risk of surgical site infection and shortening postoperative hospitalization. Although this technique may require additional surgical steps, the reduced incidence of SSI and lower surgical costs make it a viable option for wound closure. Further research is needed to investigate the potential benefits of this technique in larger patient populations and evaluate its long-term effectiveness.

ARTICLE HIGHLIGHTS

Research background

In the traditional intestinal stoma closure surgery, the interrupted suturing technique used has some issues, such as longer incisions and higher tension on the incision, which may increase the risk of postoperative complications. To address these concerns, scholars have proposed the use of the "gunsight suture" technique. This method involves using a gun-shaped incision instead of the conventional linear incision, leaving a gap in the center for drainage of blood and fluids to reduce the risk of infection. Building on this, we have proposed an improved gunsight suture technique.

Research motivation

Motivated by the need to enhance patient outcomes, this study focuses on developing an improved prophylactic stoma reversal abdominal closure suturing technique. Our goal is to reduce incision infection rates and alleviate patient burden, improving a key issue in stoma closure methods. We strive to contribute to the advancement of surgical research and help shape safer and more effective surgical practices in the future.

Research objectives

To compare the outcomes of the improved gunsight suture technique with traditional interrupted suture techniques in enterostomy closure. The objective is to minimize complications and related burdens following prophylactic ileostomy closure.

Research methods

This study analyzed 270 patients who underwent prophylactic ileostomy closure surgery at Qilu Hospital from April 2017 to December 2021. The patients were divided into two groups, one group received sutures using the improved gunsight method while the other group was sutured with the traditional interrupted suture method, and data on various parameters such as operation time, postoperative pain score, and hospitalization costs were collected. Non-parametric tests and chi-square tests were used for data analysis.

Research results

This retrospective study compared two suture methods for prophylactic ileostomy closure surgery in 270 patients. The modified gunsight suture group had better outcomes, including lower incidence of surgical site infections, shorter hospital stays, and fewer dressing changes than the traditional simple interrupted suture group. Laboratory parameters, postoperative temperature, pain scores, and time to first bowel gas passage did not differ significantly. The modified gunsight suture group had slightly lower surgical costs and significantly lower scar formation after incision healing.

Research conclusions

The modified Gunsight suture technique reduces the risk of surgical site infection and shortens postoperative hospitalization. Despite requiring additional surgical steps, the technique lowers surgical costs and is a viable option for wound closure.

Research perspectives

Despite the study's retrospective design and challenges in obtaining accurate data on bowel obstruction incidence and long-term complications like incisional hernias, it provides valuable insights. However, the single-center data source may introduce bias during data collection and analysis. Further research on larger patient populations is needed to evaluate the technique's long-term effectiveness and potential benefits.

FOOTNOTES

Author contributions: Chen C and Zhang X contributed equally to this work; Wang YL, Zhang X, Cheng ZQ, Zhang BB, Wang KX, and Dai Y designed the study; Chen C and Li X carried out relevant clinical work and data collection; Chen C and Zhang X analyzed the data and wrote the manuscript; and all authors have read and approved the final manuscript.

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Institutional review board statement: The study was reviewed and approved by the Ethics Committee of Qilu Hospital (Approval No. KYLL-2020-120).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Data sharing statement: The dataset generated or analyzed in this study is not available to the public. Due to privacy and ethical considerations, we are unable to provide relevant data. We will ensure compliance with relevant regulations and policies to protect the

privacy and confidentiality of participants. If you have specific questions or questions about this study, please feel free to contact the corresponding author at any time.

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ORIGINAL ARTICLE

Retrospective Study

Prevalence and risk factors of osteoporosis detected by dual-energy X-ray absorptiometry among Chinese patients with primary biliary cholangitis

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Abstract

BACKGROUND

Osteoporosis is an extrahepatic complication of primary biliary cholangitis (PBC) that increases the risk of fractures and mortality. However, Epidemiological studies of osteoporosis in patients with PBC in China and the Asia-Pacific region is lack.

AIM

To assess the prevalence and clinical characteristics of osteoporosis in Chinese patients with PBC.

METHODS

This retrospective analysis included consecutive patients with PBC from a tertiary care center in China who underwent bone mineral density (BMD) assessment using dual-energy X-ray absorptiometry between January 2013 and December 2021. We defined subjects with T-scores ≤ -2.5 in any sites (L1 to L4, femoral neck, or total hip) as having osteoporosis. Demographic, serological, clinical, and histological data were collected. Independent risk factors for osteoporosis were identified by multivariate logistic regression analysis.

RESULTS

A total of 268 patients with PBC [236 women (88.1%); mean age, 56.7 ± 10.6 years; 163 liver biopsies (60.8%)] were included. The overall prevalence of osteoporosis in patients with PBC was 45.5% (122/268), with the prevalence of osteoporosis in women and men being 47.0% and 34.4%, respectively. The prevalence of osteoporosis in postmenopausal women was significantly higher than that in premenopausal women (56.3% *vs* 21.0%, $P < 0.001$). Osteoporosis in patients with PBC is associated with age, fatigue, menopausal status, previous steroid therapy, body mass index (BMI), splenomegaly, gastroesophageal varices, ascites, Mayo

risk score, histological stage, alanine aminotransferase, albumin, bilirubin, platelet and prothrombin activity. Multivariate regression analysis identified that older age, lower BMI, previous steroid therapy, higher Mayo risk score, and advanced histological stage as the main independent risk factors for osteoporosis in PBC.

CONCLUSION

Osteoporosis is very common in Chinese patients with PBC, allowing for prior screening of BMD in those PBC patients with older age, lower BMI, previous steroid therapy and advanced liver disease.

Key Words: Primary biliary cholangitis; Osteoporosis; Bone mineral density; Dual-energy X-ray absorptiometry; Prevalence; Chinese

Core Tip: In this paper, we reported for the first large-sample study to explore the prevalence and potential risk factors for osteoporosis in Chinese patients with primary biliary cholangitis (PBC). The prevalence of osteoporosis in Chinese patients with PBC was 45.5%. Osteoporosis in PBC is strongly associated with older age, lower body mass index, previous steroid use, the severity of liver disease, and advanced histological stage. This study provides reference information for future PBC-related guideline development and public policy formulation in China and the Asia-Pacific region.

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INTRODUCTION

Primary biliary cholangitis (PBC, also known as primary biliary cirrhosis) is a chronic immune-mediated, progressive cholestatic liver disease characterized by nonsuppurative destructive intrahepatic cholangitis focused on the small bile ducts, which mainly affects middle-aged women over 40 years old, typically manifested as fatigue, pruritus and even metabolic bone disease[1,2]. Currently, ursodeoxycholic acid (UDCA) is its first-line treatment drug, and untreated PBC may eventually lead to cirrhosis and liver failure, requiring liver transplantation[3]. Moreover, PBC affects all races and ethnicities with significant regional differences[1,4]. It is estimated that at least 1 in 1000 women older than 40 years globally has PBC[5], and the prevalence and incidence of PBC in Europe and the United States are higher than that in the Asia-Pacific region[6]. However, the prevalence of PBC in China ranks second only to Japan in the Asia-Pacific region and is increasing[6].

Osteoporosis is a disease characterized by decreased bone density or bone loss, leading to an increased risk of fracture [7]. The prevalence of osteoporosis in patients with PBC is at least three times that of age- or sex-matched controls[8,9]. Thus, osteoporosis is considered an extrahepatic complication of PBC[10]. Unlike increased bone resorption in postmenopausal osteoporosis, osteoporosis in PBC is mainly caused by decreased bone formation[11]. Moreover, recently, a large cohort study indicated that the risk of fracture and post-fracture mortality of PBC patients were significantly higher than those of the control group in the general population[12]. Prevention and timely diagnosis of osteoporosis are key to reducing the associated complications for patients with PBC. With the increasing prevalence of PBC, most likely due to the improvement of diagnosis and awareness, it is likely that the incidence and prevalence of PBC-related osteoporosis will also increase[11]. According to previous studies from Europe, Africa and North America, the prevalence of osteoporosis in patients with PBC is approximately 30% (range 20%-52%) and higher in postmenopause or advanced stages of liver disease[8,9,13-18], and up to 44% of patients waiting for liver transplantation[16]. However, to our knowledge, in China and other Asia–Pacific regions, the prevalence and clinical features of osteoporosis in patients with PBC, including men and women, have not been well defined.

Therefore, this study aimed to investigate the prevalence and clinical features of osteoporosis and independent risk factors associated with osteoporosis using bone mineral density (BMD) detected by dual-energy X-ray absorptiometry (DEXA) in a large series of Chinese patients with PBC.

MATERIALS AND METHODS

Study design

We carried out a retrospective observational study of all consecutive PBC patients between January 2013 and December 2021 from Beijing Ditan Hospital, China. The baseline date was the first admission with a diagnosis of PBC and completion of a BMD examination in the hospital. The study protocol was approved by the Ethics Committee of Beijing

Ditan Hospital, Beijing, China (No. DTEC-KT2022-010-01).

The inclusion criteria were as follows: (1) Age greater than 18 years old; (2) at least two of the following: Elevated γ-glutamyl transpeptidase (GGT) or alkaline phosphatase (ALP), positive antimitochondrial antibody (AMA) or gp210, sp100, and pathological features of non-suppurative cholangitis or small bile duct destruction[19]; and (3) complete BMD examination using DEXA method at baseline. The exclusion criteria were as follows: (1) Alcoholic liver disease, non-alcoholic fatty liver disease, viral hepatitis, drug-induced liver injury, or inherited liver disease; (2) liver transplantation, liver cancer, or other malignant lesions; (3) evidence of intrahepatic or extrahepatic biliary obstruction; (4) severe cardiac or renal insufficiency; (5) previous or current hormone replacement therapy; and (6) pregnancy or breast-feeding.

Data collection

A history of UDCA or steroid treatment, prior fractures, and comorbidities were recorded. Symptoms of chronic cholestasis, such as fatigue and pruritus, and physical signs of liver disease, such as splenomegaly, hepatomegaly, edema and ascites, were collected. Blood was collected for hematological, biochemical and immunological tests after an overnight fast and tested at the Laboratory of Beijing Ditan Hospital using standard methods. These laboratory parameters include serum bilirubin, albumin, ALP, GGT, calcium, phosphorus, creatinine, 25-hydroxyvitamin D, aspartate aminotransferase, alanine aminotransferase (ALT), prothrombin activity (PTA), platelet count (PLT), immunoglobulin G, serum immunoglobulin M, and autoantibodies [AMA, antinuclear antibody (ANA), anti-centromere antibody (ACA), gp210, and sp100] were measured at the time of the first BMD examination. Age, smoking status, body mass index (BMI), menopausal status, duration of PBC, histological stage, and cirrhosis status were recorded. Esophagogastroscopy and abdominal ultrasonography were also evaluated. The Mayo risk score (MRS) was calculated using the previous algorithm[20]. The liver histological stage was determined according to Ludwig's criteria[21].

BMD measurement

Certified technicians measured BMD at the lumbar spine (L1 to L4), femoral neck, and total hip using a DEXA scanner (Lunar, GE Healthcare, United States). The diagnoses of osteopenia and osteoporosis were based on the World Health Organization thresholds: T-score is between -1.0 and -2.5 and ≤ -2.5, respectively[22]. T-score were presented as absolute values (g/cm^2) and the number of SD lower than the average peak value of young sex-matched normal individuals. Z-score are also presented as the number of SD from normal values corrected for sex and age. We defined patients with T-scores ≤ -2.5 in any sites (L1 to L4, femoral neck, or total hip) as having osteoporosis[23].

Statistical analysis

All statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, United States). Quantitative data are expressed as mean ± SD or median and interquartile range, and categorical data are expressed as frequencies with percentages. Student *t*-test or Mann-Whitney U test was used to analyse differences in continuous variables. Chi-squared or Fisher's exact tests were used for categorical variables. The independent risk factor of significant variable associated with osteoporosis with (P value < 0.05) in univariate analyses was determined using multivariate logistic regression with the backward stepwise selection method (the criteria for entering and removing variables were P < 0.05 and > 0.10, respectively). Statistical significance was defined as a two-tailed P value < 0.05.

RESULTS

Demographic and clinical characteristics

From January 2013 to December 2021, we retrospectively enrolled 268 subjects in the final analysis who had undergone BMD examination by DEXA scanner from 1272 patients with PBC. Figure 1 summarizes the enrolment process. The demographic, clinical, serological, histological, and BMD characteristics of all patients with PBC are shown in Table 1. The mean age of the overall patients was 56.7 ± 10.6 years (range 29-94 years). The ratio of women:men was 7.38:1. Liver biopsies were performed in 60.8% of patients. Among the women, 73.7% were postmenopausal. Prior fractures had occurred in 11 patients, including six vertebral fractures and five peripheral fractures. Compared with women, men PBC patients had more smoker, UDCA-treated patients, fewer ACA positive, lower serum calcium, and higher serum creatinine as well as BMD absolute value (all P < 0.05). However, there was no difference between men and women in the T- and Z-score (P > 0.05).

Prevalence of osteoporosis

There were significant differences in the BMD value, T-score, and Z-score at any sites (L1 to L4, femoral neck, or total hip) in patients with osteoporosis compared to those without (all P < 0.001) (Supplementary Table 1). The prevalence of osteoporosis and osteopenia in the lumbar spine (L1 to L4), femoral neck and total hip were 40.3% and 35.8%, 29.1% and 45.9%, 16.8% and 47.4%, respectively (Table 1). Overall, the prevalence of osteoporosis was 45.5% (122/268) in all PBC patients, considering the lowest BMD values at the lumbar spine, femoral neck and total hip (Table 1 and Supplementary Table 2). The prevalence of osteoporosis 47.0% (111/236) was higher among women than among men 34.4% (11/32), but it was not statistically significant (P = 0.177) (Supplementary Table 2 and Figure 2). The prevalence of osteoporosis in postmenopausal women was significantly higher than that in premenopausal women (56.3% *vs* 21.0%, P < 0.001) (Supplementary Table 2 and Figure 2). The prevalence of osteoporosis was assessed according to age, cirrhosis status, histological stage and BMI. Regardless of overall patients or only women patients, the prevalence of osteoporosis

Table 1 Demographic, clinical, laboratory, and bone mineral density data in patients with primary biliary cholangitis

Characteristics	Overall (*n* = 268)	Men (*n* = 32)	Women (*n* = 236)	*P* value
Age (years) (range)	56.7 ± 10.6 (29-94)	60.0 ± 9.3 (38-79)	56.3 ± 10.7 (29-94)	0.059
Age group (years), *n* (%)				0.201
29-39	13 (4.9)	1 (3.1)	12 (5.1)	
40-49	53 (19.8)	2 (6.3)	51 (21.6)	
50-59	93 (34.7)	11 (34.4)	82 (34.7)	
60-69	77 (28.7)	12 (37.5)	65 (27.5)	
≥ 70	32 (11.9)	6 (18.8)	26 (11.0)	
Duration of PBC (years)	3.0 (1.0-6.0)	4.0 (1.6-6.8)	3.0 (1.0-6.0)	0.246
Smoking, *n* (%)	20 (7.5)	17 (53.1)	3 (1.3)	< 0.001
Postmenopausal, *n* (%)	174 (73.7)		174 (73.7)	
Pruritus, *n* (%)	45 (16.8)	4 (12.5)	41 (17.4)	0.489
Fatigue, *n* (%)	91 (34.0)	10 (31.3)	81 (34.3)	0.731
Hepatomegaly, *n* (%)	9 (3.4)	1 (3.1)	8 (3.4)	1.000
Splenomegaly, *n* (%)	187 (69.8)	20 (62.5)	167 (70.8)	0.340
Gastroesophageal varices, *n* (%)	110 (41.0)	14 (43.8)	96 (40.7)	0.740
Ascites, *n* (%)	103 (38.4)	11 (34.4)	92 (39.0)	0.615
Prior fractures, *n* (%)	11 (4.1)	0 (0.0)	11 (4.7)	0.371
BMI (kg/m^2)	22.6 ± 3.3	22.1 ± 3.0	22.7 ± 3.4	0.367
Comorbidities				
Diabetes mellitus, *n* (%)	46 (17.2)	8 (25.0)	38 (16.1)	0.210
Hypertension, *n* (%)	58 (21.6)	7 (21.9)	51 (21.6)	0.973
Hashimoto's thyroiditis, *n* (%)	66 (24.6)	7 (21.9)	59 (25.0)	0.700
Rheumatoid arthritis, *n* (%)	8 (3.0)	0 (0.0)	8 (3.4)	0.602
Sicca syndrome, *n* (%)	18 (6.7)	1 (3.1)	17 (7.2)	0.706
Previous medication, *n* (%)				
UDCA use	155 (57.8)	24 (75.0)	131 (55.5)	0.036
Steroid use	32 (11.9)	3 (9.4)	29 (12.3)	0.633
Cirrhosis status, *n* (%)				0.661
Non-cirrhosis	107 (39.9)	15 (46.9)	92 (39.0)	
Compensated	53 (19.8)	5 (15.6)	48 (20.3)	
Decompensated	108 (40.3)	12 (37.5)	96 (40.7)	
PBC-AIH overlap syndrome, *n* (%)	42 (15.7)	5 (15.6)	37 (15.7)	0.994
Mayo risk score	5.2 ± 1.4	5.4 ± 1.4	5.1 ± 1.5	0.301
Histological stage, *n* (%)[1]				0.630
I-II	65 (39.9)	6 (46.2)	59 (39.3)	
III-IV	98 (60.1)	7 (53.8)	91 (60.7)	
Laboratory data				
ALT (U/L)	36.2 (22.2-70.0)	42.8 (29.0-74.4)	35.8 (21.3-69.7)	0.236
AST (U/L)	46.4 (29.2-88.7)	39.2 (30.1-62.2)	48.1 (29.0-87.7)	0.642
Bilirubin (mg/dL)	1.1 (0.7-1.9)	0.9 (0.7-1.5)	1.1 (0.7-2.0)	0.708
Albumin (g/L)	37.2 ± 6.6	35.5 ± 6.0	37.4 ± 6.6	0.124

ALP (U/L)	157.2 (101.7-263.1)	166.3 (102.8-315.8)	156.5 (101.2-262.4)	0.747
GGT (U/L)	118.2 (48.0-276.3)	146.3 (52.3-321.2)	117.0 (47.6-276.3)	0.568
PTA (%)	93.0 ± 20.6	92.6 ± 19.9	93.0 ± 20.8	0.922
PLT (× 10^9/L)	140.2 ± 79.4	140.7 ± 80.1	136.2 ± 74.4	0.761
Calcium (mmol/L)	2.22 ± 0.14	2.17 ± 0.16	2.23 ± 0.14	0.018
Phosphorous (mmol/L)	1.14 ± 0.24	1.09 ± 0.17	1.15 ± 0.25	0.201
25-hydroxyvitamin D (ng/mL)[2]	14.3 ± 6.9	17.2 ± 6.6	13.6 ± 6.9	0.224
Creatinine (μmol/L)	56.4 (49.5-65.9)	67.1 (60.2-86.6)	55.0 (48.6-63.6)	< 0.001
IgM (g/L)	2.86 (1.60-4.40)	2.55 (1.16-4.14)	2.87 (1.64-4.49)	0.240
IgG (g/L)	15.3 (12.0-19.1)	15.0 (11.9-19.3)	15.6 (12.0-19.1)	0.674
gp210 (+), *n* (%)	99 (36.9)	10 (31.3)	89 (37.7)	0.477
Sp100 (+), *n* (%)	38 (14.2)	1 (12.5)	34 (14.4)	0.772
ACA, *n* (%)	54 (20.1)	2 (6.3)	52 (22.0)	0.037
ANA, *n* (%)	165 (61.6)	21 (65.6)	144 (61.0)	0.615
Lumbar spine BMD (L1-L4), g/cm^2	0.828 ± 0.142	0.893 ± 0.146	0.818 ± 0.139	0.005
T-score	-2.04 ± 1.27	-1.81 ± 1.31	-2.1 ± 1.27	0.267
Z-score	-0.929 ± 1.182	-1.18 ± 1.33	-0.89 ± 1.16	0.198
BMD classification, *n* (%)				0.536
Osteoporosis	108 (40.3)	10 (31.3)	98 (41.5)	
Osteopenia	96 (35.8)	13 (40.6)	83 (35.2)	
Normal	64 (23.9)	9 (28.1)	55 (23.3)	
Femoral neck BMD, g/cm^2	0.654 ± 0.128	0.714 ± 0.139	0.646 ± 0.125	0.005
T-score	-1.80 ± 1.12	-1.59 ± 1.03	-1.83 ± 1.13	0.262
Z-score	-0.73 ± 1.02	-0.63 ± 0.99	-0.74 ± 1.03	0.590
BMD classification, *n* (%)				0.374
Osteoporosis	78 (29.1)	7 (21.9)	71 (30.1)	
Osteopenia	123 (45.9)	14 (43.8)	109 (46.2)	
Normal	67 (25.0)	11 (34.4)	56 (23.7)	
Total Hip BMD, g/cm^2	0.784 ± 0.153	0.855 ± 0.148	0.774 ± 0.152	0.005
T-score	-1.42 ± 1.06	-1.19 ± 0.98	-1.45 ± 1.07	0.198
Z-score	-0.64 ± 0.98	-0.70 ± 0.96	-0.63 ± 1.00	0.714
BMD classification, *n* (%)				0.601
Osteoporosis	45 (16.8)	5 (15.6)	40 (16.9)	
Osteopenia	127 (47.4)	13 (40.6)	114 (48.3)	
Normal	96 (35.8)	14 (43.8)	82 (34.7)	
Osteoporosis lumbar or neck or hip	122 (45.5)	11 (34.4)	111 (47.0)	0.177

[1]Available in 163 patients.
[2]Available in 46 patients.
Data are presented as mean ± SD or median (interquartile range) or *n* (%). ACA: Anti-centromere antibody; AIH: Autoimmune hepatitis; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; ANA: Antinuclear antibody; AST: Aspartate aminotransferase; BMD: Bone mineral density; BMI: Body mass index; GGT: γ-glutamyl transpeptidase; IgG: Immunoglobulin G; IgM: Immunoglobulin M; PBC: Primary biliary cholangitis; PLT: Platelet count; PTA: Prothrombin activity; UDCA: Ursodeoxycholic acid.

increased with age, cirrhosis status, and histological stage, but decreased with BMI (all *P* for trend < 0.001) (Supplementary Table 2, Figures 2 and 3).

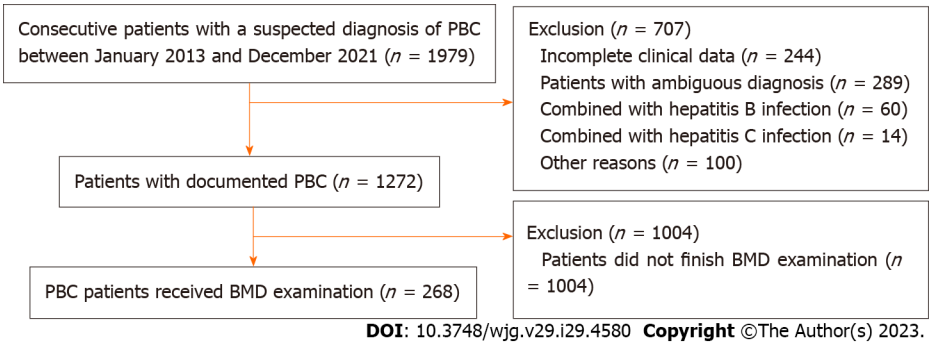

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Figure 1 The flowchart of patient enrolment. PBC: Primary biliary cholangitis; BMD: Bone mineral density.

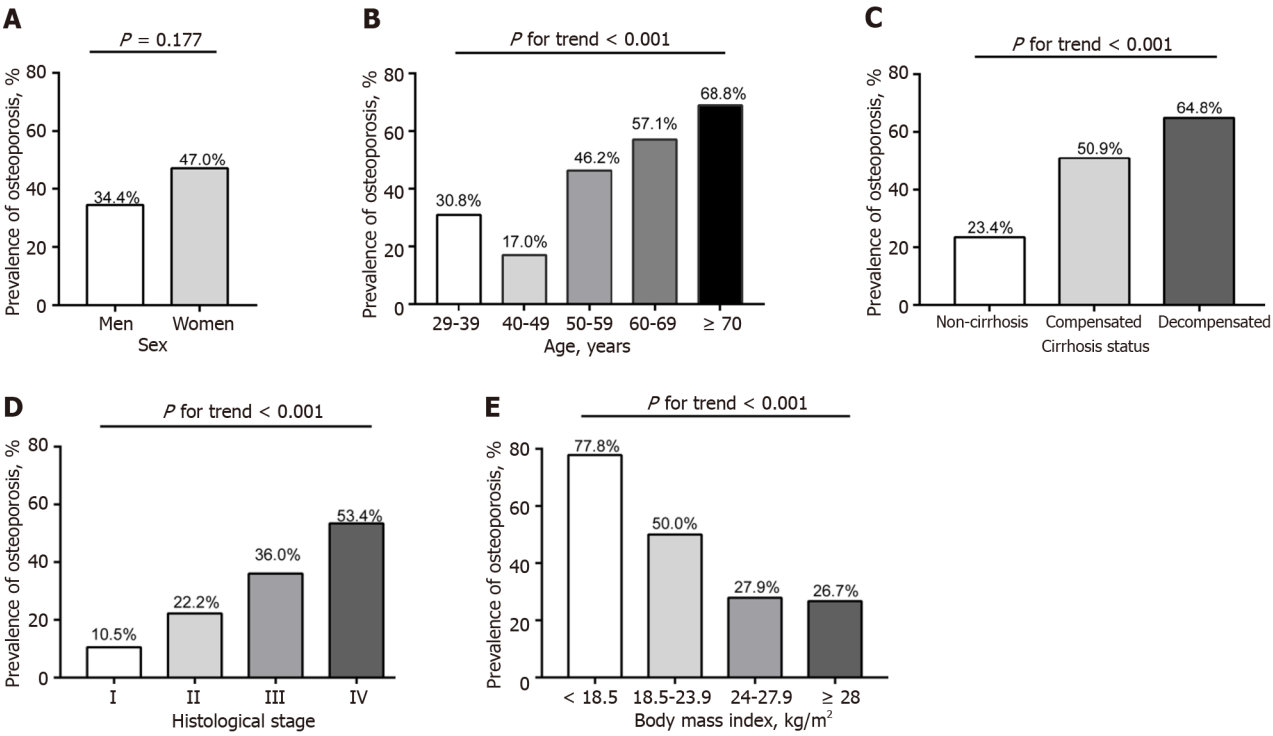

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Figure 2 Prevalence of osteoporosis in different subgroups for overall patients with primary biliary cholangitis (*n* = 268). A: Sex; B: Age; C: Cirrhosis status; D: Histological stage; E: Body mass index.

Risk factors of osteoporosis

Table 2 shows the results of univariate analysis of the association between osteoporosis and potential risk factors in patients with PBC. In the univariate analysis, osteoporosis was correlated with older age, fatigue, postmenopausal status, previous steroids use, splenomegaly, gastroesophageal varices, ascites, advanced histological stage (III or IV), higher MRS and bilirubin levels, and lower BMI, ALT, albumin, PTA, and PLT regardless of overall or women patients (all $P < 0.05$). However, there was no association between osteoporosis and other biochemical parameters or immunological indicators such as immunoglobulin, gp210, sp100, ACA, PBC-autoimmune hepatitis (AIH) overlap syndrome, or extrahepatic autoimmune diseases.

In multivariate analysis, older age [odds ratio (OR), 1.80; 95%CI (confidence interval): 1.33-2.44, $P < 0.001$] per 10 years, gastroesophageal varices (OR, 2.11; 95%CI: 1.14-3.92, $P = 0.018$), lower BMI (OR, 0.85; 95%CI: 0.77-0.93, $P < 0.001$), previous steroid use (OR, 4.19; 95%CI: 1.66-10.56, $P = 0.002$), higher MRS (OR, 1.36; 95%CI: 1.08-1.71, $P = 0.009$) were the independent risk factors associated with the presence of osteoporosis in all patients with PBC (Table 3). In addition, when the histological stage was included in the multivariate analysis, higher bilirubin (OR, 1.20; 95%CI: 1.0-1.42, $P = 0.044$) and advanced histological stage (OR, 3.74; 95%CI: 1.60-8.77, $P = 0.002$) gained statistical significance, but the effects of gastroesophageal varices, previous steroid use, and higher MRS were removed. When menopausal status was included in the multivariate analysis (only for female patients), the splenomegaly (OR, 2.62; 95%CI: 1.18-5.80, $P = 0.018$) and postmenopausal (OR, 2.92; 95%CI: 1.02-8.38, $P = 0.046$) gained statistical significance. When both menopausal status and histological stage were included in the multivariate analysis, advanced histological stage, older age, lower BMI, previous

Table 2 Univariate analysis of risk factors for osteoporosis in overall and women patients with primary biliary cholangitis

Variables	Overall patients (*n* = 268)		*P* value	Women patients (*n* = 236)		*P* value
	Osteoporosis (*n* = 122)	No osteoporosis (*n* = 146)		Osteoporosis (*n* = 111)	No osteoporosis (*n* = 125)	
Women, *n* (%)	111 (91.0)	125 (85.6)	0.177	111 (100)	125 (100)	
Age (year)	60.5 ± 10.5	53.6 ± 9.7	< 0.001	60.4 ± 10.7	52.6 ± 9.4	< 0.001
Duration of PBC (year)	3.0 (1.0-7.0)	2.3 (1.0-5.0)	0.125	3.0 (1.0-7.0)	2.0 (1.0-5.0)	0.217
Smoking, *n* (%)	9 (7.4)	11 (7.5)	0.961	2 (1.8)	1 (1.6)	0.602
Postmenopausal, *n* (%)	98 (88.3)	76 (60.8)	< 0.001	98 (88.3)	76 (60.8)	< 0.001
Pruritus, *n* (%)	22 (18.0)	23 (15.8)	0.619	21 (18.9)	20 (16.0)	0.555
Fatigue, *n* (%)	50 (41.0)	41 (28.1)	0.026	48 (43.2)	33 (26.4)	0.007
Hepatomegaly, *n* (%)	2 (1.6)	7 (4.8)	0.188	2 (1.8)	6 (4.8)	0.204
Splenomegaly, *n* (%)	103 (84.4)	84 (57.5)	< 0.001	96 (86.5)	71 (56.8)	< 0.001
Gastroesophageal varices, *n* (%)	72 (59.0)	38 (26.0)	< 0.001	66 (59.5)	30 (24.0)	< 0.001
Ascites, *n* (%)	67 (54.9)	36 (24.7)	< 0.001	61 (55.0)	31 (24.8)	< 0.001
Prior fractures, *n* (%)	8 (5.0)	3 (2.1)	0.064	8 (7.2)	3 (2.4)	0.080
BMI (kg/m^2)	21.6 ± 3.2	23.4 ± 3.3	< 0.001	21.8 ± 3.1	23.4 ± 3.4	< 0.001
Comorbidities						
Diabetes mellitus, *n* (%)	23 (18.9)	23 (15.8)	0.503	20 (18.0)	18 (14.4)	0.450
Hypertension, *n* (%)	28 (23.0)	30 (20.5)	0.634	26 (23.4)	25 (20.0)	0.524
Hashimoto's thyroiditis, *n* (%)	27 (22.1)	39 (26.7)	0.386	26 (23.4)	33 (26.4)	0.598
Rheumatoid arthritis, *n* (%)	4 (3.3)	4 (2.7)	0.796	4 (3.6)	4 (3.2)	0.864
Sicca syndrome, *n* (%)	8 (6.6)	10 (6.8)	0.924	8 (7.2)	9 (7.2)	0.998
Previous medication						
UDCA use, *n* (%)	78 (63.9)	77 (52.7)	0.065	69 (62.2)	62 (49.6)	0.053
Steroid use, *n* (%)	23 (18.9)	9 (6.2)	0.001	21 (18.9)	8 (6.4)	0.003
PBC-AIH overlap syndrome, *n* (%)	17 (13.9)	25 (17.1)	0.475	17 (15.3)	20 (16.0)	0.885
Mayo risk score	5.7 ± 1.5	4.7 ± 1.3	< 0.001	5.7 ± 1.4	4.6 ± 1.2	< 0.001
Histological stage, *n* (%)[1]			< 0.001			< 0.001
I-II	10 (17.2)	55 (52.4)		9 (16.1)	50 (53.2)	
III-IV	48 (82.8)	50 (47.6)		47 (83.9)	44 (46.8)	
ALT (U/L)	32.0 (20.8-54.8)	43.7 (23.7-84.4)	0.007	31.2 (20.5-31.2)	46.3 (29.1-84.4)	0.013
AST (U/L)	44.0 (29.1-83.7)	49.5 (29.2-91.5)	0.782	46.3 (29.1-84.4)	50.9 (28.7-91.5)	0.899
Bilirubin (mg/dL)	1.2 (0.8-2.40	0.9 (0.7-1.7)	0.002	1.2 (0.8-2.5)	0.9 (0.7-1.6)	0.001
Albumin (g/L)	35.5 ± 6.9	38.7 ± 5.9	< 0.001	35.7 ± 6.9	39.0 ± 5.9	< 0.001
ALP (U/L)	164.2 (110.8-266.3)	149.3 (94.4-261.5)	0.180	164.3 (112.3-265.0)	144.5 (93.6-257.4)	0.175
GGT (U/L)	107.7 (47.9-237.1)	143.1 (47.7-307.3)	0.320	108.5 (45.4-242.5)	129.1 (48.6-303.3)	0.432
PTA (%)	87.9 ± 20.2	97.2 ± 20.1	< 0.001	87.6 ± 20.2	97.8 ± 20.1	< 0.001
PLT (× 10^9/L)	119.3 ± 71.4	157.7 ± 81.7	< 0.001	116.4 ± 67.4	162.4 ± 84.5	< 0.001
Calcium (mmol/L)	2.20 ± 0.15	2.24 ± 0.13	0.065	2.21 ± 0.14	2.24 ± 0.13	0.100
Phosphorous (mmol/L)	1.15 ± 0.30	1.14 ± 0.17	0.703	1.15 ± 0.31	1.15 ± 0.17	0.869

25-hydroxyvitamin D (ng/mL)[2]	14.0 ± 7.8	14.5 ± 6.2	0.823	14.1 ± 8.0	13.4 ± 5.8	0.748
Creatinine (μmol/L)	58.4 (47.2-67.5)	56.2 (50.6-65.4)	0.724	55.9 (46.9-67.5)	54.9 (49.9-60.7)	0.710
IgM (g/L)	2.80 (1.53-4.39)	2.87 (1.64-4.63)	0.497	2.87 (1.61-4.49)	2.93 (1.65-4.49)	0.776
IgG (g/L)	15.7 (12.0-19.5)	15.2 (11.9-18.9)	0.453	15.7 (12.3-19.6)	15.3 (11.8-18.6)	0.420
gp210 (+), *n* (%)	48 (39.3)	51 (34.9)	0.456	45 (40.5)	44 (35.2)	0.398
Sp100 (+), *n* (%)	19 (15.6)	19 (13.0)	0.550	18 (16.2)	16 (12.8)	0.456
ACA, *n* (%)	30 (24.6)	24 (16.4)	0.098	29 (26.1)	23 (18.4)	0.153
ANA, *n* (%)	74 (60.7)	91 (62.3)	0.779	69 (62.2)	75 (60.0)	0.734

[1]Available in 163 patients.
[2]Available in 46 patients.
Data are presented as mean ± SD or median (interquartile range) or *n* (%). ACA: Anti-centromere antibody; AIH: Autoimmune hepatitis; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; ANA: Antinuclear antibody; AST: Aspartate aminotransferase; BMI: Body mass index; GGT: γ-glutamyl transpeptidase; IgG: Immunoglobulin G; IgM: Immunoglobulin M; PBC: Primary biliary cholangitis; PLT: Platelet count; PTA: Prothrombin activity; UDCA: Ursodeoxycholic acid.

Table 3 Multivariate analysis of risk factors for osteoporosis in overall and women patients with primary biliary cholangitis

Variables	Overall patients (*n* = 268)		Overall patients underwent liver biopsy (*n* = 163)		Women patients (*n* = 236)		Women patients underwent liver biopsy (*n* = 150)	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age, per 10 years	1.80 (1.33-2.44)	< 0.001	1.95 (1.30-2.92)	0.001	1.63 (1.04-2.57)	0.035	2.02 (1.27-3.20)	0.003
Fatigue		NS		NS		NS		NS
Splenomegaly		NS		NS	2.62 (1.18-5.80)	0.018		NS
Gastroesophageal varices	2.11 (1.14-3.92)	0.018		NS		NS		NS
Ascites		NS		NS		NS		NS
BMI	0.85 (0.77-0.93)	< 0.001	0.87 (0.77-0.98)	0.023	0.83 (0.75-0.92)	< 0.001	0.85 (0.74-0.97)	0.014
Previous steroid use	4.19 (1.66-10.56)	0.002		NS	4.01 (1.42-11.31)	0.009	3.99 (1.24-12.87)	0.020
Mayo risk score	1.36 (1.08-1.71)	0.009		NS	1.61 (1.22-2.14)	0.001	1.64 (1.12-2.41)	0.011
ALT		NS		NS		NS		NS
Bilirubin		NS	1.20 (1.01-1.42)	0.044		NS		NS
Albumin		NS		NS		NS		NS
PTA		NS		NS		NS		NS
PLT		NS		NS		NS		NS
Postmenopausal					2.92 (1.02-8.38)	0.046		NS
Histological stage III or IV			3.74 (1.60-8.77)	0.002			3.02 (1.13-8.06)	0.027

ALT: Alanine aminotransferase; BMI: Body mass index; CI: Confidence interval; NS: Not significant; OR: Odds ratio; PLT: Platelet count; PTA: Prothrombin activity.

steroid use, and higher MRS were identified as independent risk factors for osteoporosis. However, menopausal status and other variables, such as bilirubin, were not entered as independent factors of osteoporosis in the final model.

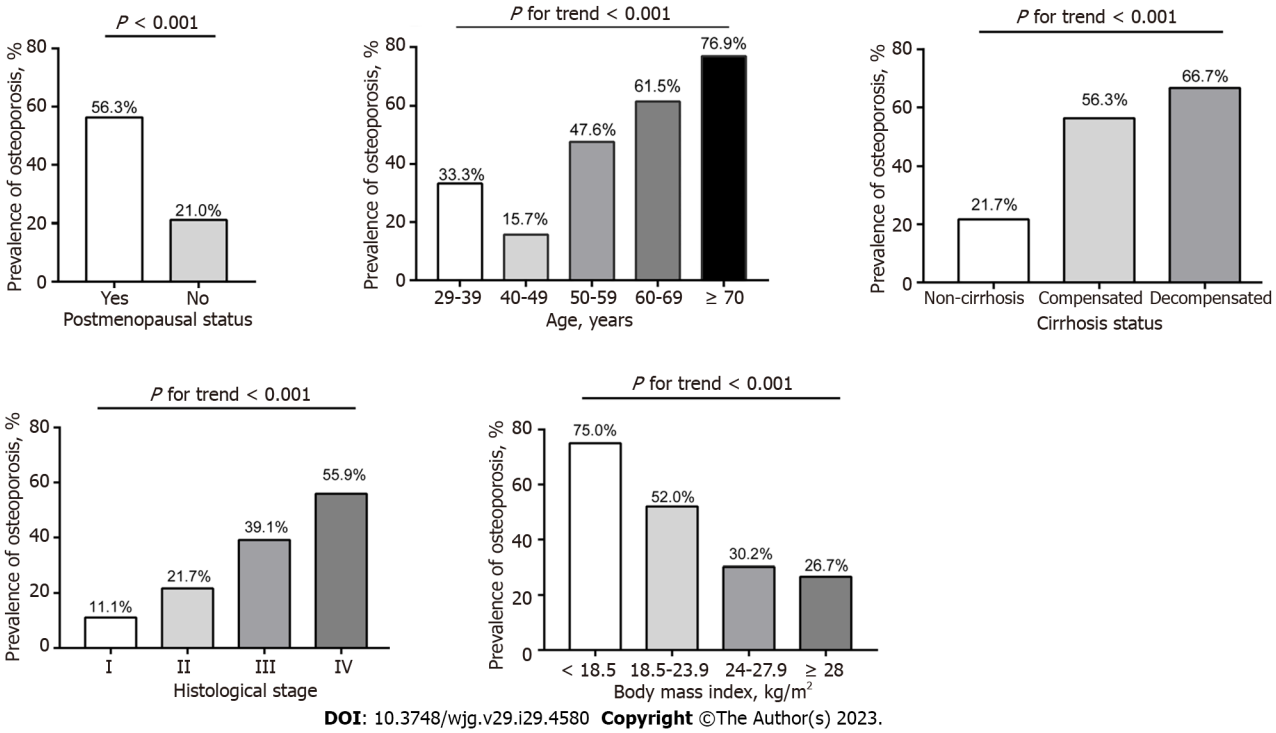

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Figure 3 Prevalence of osteoporosis in different subgroups for women patients with primary biliary cholangitis (*n* = 236). A: Postmenopausal status; B: Age; C: Cirrhosis status; D: Histological stage; E: Body mass index.

DISCUSSION

To our knowledge, this observational study is the largest population study on BMD in PBC among currently published studies, whether in China, the Asia-Pacific region, or globally. Moreover, this is the first large-sample study to explore the prevalence and potential risk factors for osteoporosis in Asian patients with PBC. PBC patients with advanced stage (Ludwig III/IV) had more than 2-fold increased risk of osteoporosis compared to patients with early stage (Ludwig I/II). The main independent risk factors identified for osteoporosis include older age, lower BMI, previous steroid use, liver disease severity determined by the MRS, and advanced histological stage. These factors are consistent with those of previous studies in Europe and the United States[8,9].

A recent meta-analysis indicated that the risk of osteoporosis increased by 1.8 times in PBC patients compared with non-PBC participants[24]. Similar to this result, our study indicated a 2.3-time increased risk of osteoporosis in PBC patients aged 40 years or older, accounting for 95% of the total patients, compared to age-matched controls in China[23]. However, by searching the literature, we found that since 2001, the prevalence of PBC osteoporosis evaluated by DEXA has been verified in European, African, and American populations but not in Asian populations (Supplementary Table 3). As is well known, genetic factors play a crucial role in the pathogenesis of PBC[1], it is imperative to explore the prevalence of PBC osteoporosis in Asian populations. The findings of our study fill the gap in the epidemiological data on PBC-related osteoporosis in China and Asia. Notably, in our study, the prevalence of osteoporosis (45.5%) in PBC patients was higher than that of PBC patients in Europe and America (approximately 30%). The reason for this difference may be ethnic differences or a high proportion of patients with cirrhosis (60.1%) or decompensated cirrhosis (40.3%) in our study. However, the prevalence of osteoporosis in noncirrhotic PBC patients (23.4%) in our study was similar to that reported in Europe and the United States.

It has been recognized that glucocorticoid administration can significantly increase the risk of osteoporosis and bone fragility[25]. Consistently, our study found that the osteoporosis rate of PBC patients previously treated with steroid was significantly higher than that of patients without steroid treatment (71.9% *vs* 41.0%, $P = 0.001$). However, although many patients in our study were treated with UDCA (64%), it had no effect on the prevalence of osteoporosis. Previous studies have also shown that the treatment of PBC itself has not been shown to improve BMD[10]. In addition, PBC-related osteoporosis is strongly correlated with the severity of liver disease[8,9,14]. Our study results also showed that the more severe liver disease determined by MRS and histological stage in PBC, the more prone the individual is to osteoporosis. Moreover, the univariate analysis in this study demonstrated that osteoporosis in PBC was related to fatigue, higher bilirubin, lower albumin, PTA, and features of portal hypertension, including splenomegaly, gastroesophageal varices, ascites and thrombocytopenia, which are also common clinical indicators of liver disease severity. Moreover, although the pathogenesis of hepatic osteodystrophy has not been clarified, it is generally believed that chronic cholestasis itself may lead to bone loss in PBC patients[14]. Bilirubin inhibits the function of osteoblasts in vitro, which may be related to the low bone formation rate of PBC patients[26]. In our study, bilirubin levels were also statistically significant when the histological stage was considered as a variable in multivariate analysis. Similarly, Menon *et al*[9] found that higher baseline bilirubin level rather than the histological stage was the only variable independently related to bone loss rate

after 3 years of follow up[9].

In addition, lower BMI and older age are recognized risk factors for osteoporosis in postmenopausal women as well as in the general population[27]. Our study also verified the association between lower BMI and older age in PBC and osteoporosis, thus further proving that this may be associated with similar pathogenesis in the general population. However, although the menopausal status was not statistically significant when including histological stage in our multivariate analysis, it was selected as an independent indicator of osteoporosis when excluding histological stage from the model, indicating that histological stage captured the impact of menopausal status when the two variables competed in the model. Interestingly, lower ALT levels were also found to be related to a higher osteoporosis rate in the current univariate analysis, which is similar to our previous study that indicated that the biochemical response rate of PBC patients with lower ALT levels at baseline were worse than that of patients with higher ALT levels[28]. However, the ALT level was not an independent factor of osteoporosis in multivariate analysis.

In addition, one advantage of our study was that it explored the correlation between immune indicators and PBC osteoporosis, which has not been discussed in previous studies. Anti-gp210 and sp100 antibodies are two specific ANAs for PBC diagnosis[2]. Previous studies showed that gp210-positive is associated with poor prognosis in PBC patients[29]. Meanwhile, PBC is an immune-mediated cholangitis with complex pathogenesis, which often occurs concomitantly with PBC-AIH overlap syndrome and other extrahepatic autoimmune diseases such as sicca syndrome, rheumatoid arthritis, and Hashimoto's thyroiditis[2,30]. However, our study showed that osteoporosis in PBC patients was not related to these immunological features.

Up to now, the pathogenesis of PBC osteoporosis is still unclear. Most experts believed that it seems to be mainly caused by reduced bone formation, although increased bone resorption may play a role in certain situations, such as in post-menopausal women and patients with hypogonadism[10]. Osteoblast mediated bone formation and osteoclast dependent bone resorption are two opposite processes that affect bone mass: when absorption exceeds formation, bone mass will inevitably decrease, and this negative balance will lead to bone loss and osteoporosis[31]. Several studies assessing bone histomorphometry have shown that most of the osteoporosis patients with PBC had reduced tetracycline double labeling, bone formation rate, osteoblasts numbers, and reduced serum osteocalcin level, all of which indicate that osteoblast dysfunction and bone formation deficiency are the core of the pathogenesis of PBC-related osteoporosis[32-34]. In addition, other changes, increased levels of bilirubin and bile salts, and production of fibronectin may also reduce bone formation by inhibiting the proliferation and survival of osteoblasts in PBC or cholestasis[26,35]. Other conditions of PBC patients, including increased formation of osteoclast, low vitamin D levels, calcium malabsorption and sarcopenia, may be contributing factors to the panorama of PBC osteopathy[31,33,36,37].

Nevertheless, our study has several limitations. First, despite being the largest DEXA-based BMD measurement cohort of PBC to date, the sample size was relatively small, especially for men. Thus, it would be interesting and necessary to explore the same objective for larger sample size with a fairly balanced number of women and men. Second, this was a single-center, retrospective study. As a tertiary care center in China, our patients come from different regions of China and may not be representative of those at primary or secondary medical institutions. Prospective studies in Chinese populations may validate our findings. Third, a history of use of anti-osteoporosis treatments such as bisphosphonate, Vitamin D, and calcium supplementation was not included in this study for the analysis of the factors influencing osteoporosis. However, data on osteoporosis therapies related to PBC are insufficient and controversial, and the overall quality of evidence is low[11]. Therefore, we do not think that receiving anti-osteoporosis treatments in the past affected our results. In the future, it is necessary to conduct high-quality research and explore PBC specific therapies focused on improving bone formation.

CONCLUSION

In summary, we found a significantly higher prevalence of osteoporosis in Chinese patients with PBC. Osteoporosis in PBC is strongly associated with older age, lower BMI, previous steroid use, the severity of liver disease, and advanced histological stage. Thus, this study contributes to identifying PBC patients who require early screening for BMD, and potential interventions to diminish the risk of osteoporosis and fractures. This study may help to provide reference information for the development and formulation of future PBC-related guideline and public health policy in China and the Asia-Pacific region.

ARTICLE HIGHLIGHTS

Research background

Primary biliary cholangitis (PBC) is a chronic immune-mediated, progressive cholestatic liver disease. Osteoporosis is an extrahepatic complication of PBC that increases the risk of fractures and mortality.

Research motivation

Although the prevalence of osteoporosis in PBC is high in Europe and North America, relevant epidemiological studies of osteoporosis in patients with PBC in China and the Asia-Pacific region is lack.

Research objectives
To assess the prevalence and clinical characteristics of osteoporosis in Chinese patients with PBC.

Research methods
We performed a retrospective observational study to evaluation the prevalence and risk factors of osteoporosis in Chinese patients with PBC from a tertiary care center who underwent bone mineral density (BMD) assessment using dual-energy X-ray absorptiometry between January 2013 and December 2021. Demographic, serological, clinical, and histological data were collected. Independent risk factors for osteoporosis were identified by multivariate logistic regression analysis.

Research results
The prevalence of osteoporosis in Chinese patients with PBC was 45.5%. Osteoporosis in PBC is strongly associated with older age, lower body mass index (BMI), previous steroid use, the severity of liver disease, and advanced histological stage.

Research conclusions
Osteoporosis is very common in Chinese patients with PBC, allowing for prior screening of BMD in those PBC patients with older age, lower BMI, previous steroid therapy, and advanced liver disease.

Research perspectives
This study provides reference information for future PBC-related guideline development and public policy formulation in China and the Asia-Pacific region.

FOOTNOTES

Author contributions: Chen JL and Wang XB designed the research; Chen JL, Liu Y and Bi YF performed the research; Chen JL and Liu Y analyzed the data; Chen JL wrote the manuscript; Wang XB supervised the report; all authors reviewed and approved the final manuscript.

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Informed consent statement: As this study used anonymous and pre-existing data, the requirement for the informed consent from patients was waived.

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ORIGINAL ARTICLE

Observational Study

New objective scoring system to clinically assess fecal incontinence

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Abstract

BACKGROUND

Several scoring systems are used to assess fecal incontinence (FI), among which, the most commonly used are Wexner and Vaizey's scoring systems. However, there are significant lacunae in these scoring systems, due to which they are neither accurate nor comprehensive.

AIM

To develop a new scoring system for FI that is accurate, comprehensive, and easy to use.

METHODS

A pro forma was made in which six types of FI were included: solid, liquid, flatus, mucous, stress, and urge. The weight for each FI was determined by asking a group of patients and laypersons to give a disability score to each type of FI from 0 to 100 (0- least, 100- maximum disability). The disability was assessed on a modified EQ-5D+ (EuroQol) description system, 4D3L (4 dimensions and 3 levels) for each FI. The average score of each FI was calculated, divided by 10, and rounded off to determine the weight of each FI type. The scores for the three levels of frequency of each FI were assigned as never = 0 (No episode of FI ever),

occasional = 1 (≤ 1 episode of FI/ wk), and common = 2 (> 1 episode of FI/ wk), and was termed as frequency score. The score for each FI would be derived by multiplying the frequency score and the weight for that FI type. In the second phase of the study, a group of colorectal surgeons was asked to rank the six FI types in order of severity, and their ranking was compared with the patient and laypersons' rankings.

RESULTS

Fifty patients and 50 laypersons participated in the study. The weight was assigned to each FI (solid-8, liquid-8, urge-7, flatus-6, mucus-6, and stress-5), and an new scoring system was formulated. The maximum possible score was 80 (total incontinence), and the least 0 (no incontinence). The surgeons' ranking of FI severity did not correlate well with patients' and laypersons' rankings of FI, highlighting that surgeons and patients may perceive the severity of FI differently.

CONCLUSION

A new scoring system for FI was formulated, which was simple, logical, comprehensive, and easy to use, and eliminated previous shortcomings. Patients' and surgeons' perceptions of FI severity of FI did not correlate well.

Key Words: Feal incontinence; Scoring system; Urge; Stress; Flatus

Core tip: There are several scoring systems to assess fecal incontinence (FI), among which, the most commonly used are Wexner's, Vaizey's, and FI Severity Index scoring systems. However, there are major lacunae and shortcomings in these scoring systems, due to which, they are neither accurate and scientific nor comprehensive. We have developed a new scoring system to assess FI, which is better and more accurate than the existing scoring systems. The new system is more comprehensive and simple and easy to use, and most shortcomings of previous scoring systems have been addressed.

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INTRODUCTION

Fecal incontinence (FI) is defined as the accidental leakage of stools, flatus, or mucus and is a common gastrointestinal disorder. It affects between 1% and 16% of the population[1-7]. FI drastically impacts the quality of life, and cause embarrassment, anxiety, fear, or reluctance to go out, often leading to social isolation and the need to carry clothes or wear incontinence pads regularly[1,3,8-13]. The sensitive nature of FI makes it a taboo subject, and many people are reluctant to discuss these with family members or even doctors[2,9,14-16].

There are three scoring systems commonly used to assess FI: Cleveland Clinic or Wexner[17] (Table 1), St. Marks Hospital or Vaizey's[18] (Table 2), and FI Severity Index (FISI)[19] (Table 3). The Wexner and Vaizey scores are the most commonly used[20]. However, there are major lacunae in these scoring systems, due to which they are not an accurate reflection of incontinence level and its correlation with the quality of life.

First, the existing scoring systems are not comprehensive. Wexner scores evaluate only three parameters: solid, liquid, and flatus (gas) FI[17], and Vaizey scores include only four parameters: solid, gas, flatus, and urge (lack of ability to defer defecation for 15 min due to sudden need to defecate)[18]. FISI scores include mucus FI (leakage) as the fifth parameter to evaluate FI but do not include urge FI[19]. None of the scoring systems includes stress FI (leakage of fecal matter or flatus on increasing intraabdominal pressure like coughing, lifting weights, *etc.*) as a parameter to evaluate FI.

Stress FI is an essential independent parameter that should be included in a scoring system. The prevention of stress FI primarily depends on the proper functioning of the external anal sphincter (EAS) and intact straining puborectalis reflex [21]. The latter is a coordinated contraction of EAS and puborectalis muscle on coughing or straining. The damage to EAS and puborectalis during surgery or by peripheral neuritis due to any condition (like diabetes, *etc.*) can cause disruption of straining puborectalis reflex and lead to stress FI[22]. Stress FI is prevalent and is independent of other FIs with distinct etiopathogenesis and treatment, and therefore should be separately evaluated in all patients of FI[22].

Second, the different types of FIs are assigned the same weights in Wexner and Vaizey scoring system[17,18]. This implies that uncontrolled passage of flatus is given equal weight to uncontrolled leakage of solid motion. It does not seem logical and scientifically sound to equate and give equal weights to different types of FI. This was perhaps done for the sake of simplicity[20]. Keeping a scoring system simple and easy to use should be a priority, but it is not justified at the cost of accuracy and sacrifice of scientific principles.

Table 1 Wexner scoring

	Never	Rarely	Sometimes	Usually	Always
Solid	0	1	2	3	4
Liquid	0	1	2	3	4
Gas	0	1	2	3	4
Wears a pad	0	1	2	3	4
Lifestyle alteration	0	1	2	3	4

Rarely: < 1/mo; sometimes: < 1/wk to ≥ 1/mo; usually: < 1/d to ≥ 1/wk; always: ≥ 1/d.

Table 2 Vaizey's scoring

	Never	Rarely	Sometimes	Weekly	Daily
Solid stool incontinence	0	1	2	3	4
Liquid stool incontinence	0	1	2	3	4
Gas incontinence	0	1	2	3	4
Alteration in lifestyle	0	1	2	3	4
	No	Yes			
Need to wear a pad or plug	0	2			
Constipating medication	0	2			
Lack of ability to defer defecation for 15 min	0	4			

Never: No episodes in last 4 wk; rarely: 1 episode in last 4 wk; sometimes: ≥ 1 in last 4 wk but < 1/wk; weekly: ≥ 1/wk to < 1/d; always: ≥ 1/d.

Table 3 Fecal incontinence Severity Index scoring

	≥ 2 times/day (patient/surgeon scores)	Once/day (patient/surgeon scores)	≥ 2 times/week (patient/surgeon scores)	Once/week (patient/surgeon)	1-3 times/month (patient/surgeon scores)
Gas	12/9	11/8	8/6	6/4	4/2
Mucous	12/11	10/9	7/7	5/7	3/5
Liquid	19/18	17/16	13/14	10/13	8/10
Solid	18/19	16/17	13/16	10/14	8/11

Third, there are parameters included in the existing scoring systems that are not a direct measure of the degree or severity of FI. This leads to error (confounding bias). The same symptom is scored multiple times due to the inclusion of parameters like "Need to wear a pad"[17,18], "Alteration in lifestyle"[17,18], and "Need to take a constipating medicine"[18] in the Wexner and Vaizey scoring systems. For example, a patient having liquid or stool FI would also wear a pad daily, would have an alteration in lifestyle, and could also be taking constipating medicines to counter the symptom. This way, the patient with liquid or solid FI would be scored four times for the same symptom. These included questions are not independent types of FI but are corrective actions taken to control the symptoms of FI. So, apart from being the indirect measures, they would cause a multiplicity of scoring as the primary direct symptom (liquid or solid FI) would anyway be getting points.

Fourth, the patient perceptions were not taken into consideration while developing these scoring systems. As any FI scoring system is being developed for the patients, the latter's perception is paramount. It would be incorrect to assume that the surgeons' perception of different FIs would be similar to that of the patients. The FISI scoring system is the only one that has compared the surgeons' and patients' perceptions and found them to be different, yet they failed to formulate a simple and clear scoring system that was patient-centric[19]. There were several other discrepancies in the FISI study[23]. The sample size was small (26 surgeons and 34 patients)[19]. The forms were filled by interview as well as through mail. The standard definitions and the disability parameters on the basis of which scoring was made were not used[19]. The lack of proper statistical methods, the scoring system becoming too complex, and no proper weights being

allotted to each FI type were important shortcomings of the FISI system (Table 3). These could be the reasons that the FISI scoring system did not become popular with gastroenterologists and surgeons[19].

Due to these major lacunae in the existing scoring systems (not comprehensive, the different types of FIs were assigned the same weights, parameters included were not a direct measure of the degree or severity of FI, the patient perceptions were not taken into consideration while developing these scoring systems, *etc.*), a need was felt to develop a new scoring system that would be comprehensive, based primarily on patients' and laypersons' perceptions, was scientifically sound, accurate (free of bias or overlapping parameters), faithfully reflected the degree of disability, and yet would be simple and easy to use. Against this background, this study was done in two phases. In the first phase, a new scoring system was developed based on patients' and laypersons' perceptions, and in the second phase, it was analyzed to determine whether patients' perception and surgeons' assessments of the disability of different FIs were similar or not.

MATERIALS AND METHODS

The study was designed in two phases. In the first phase, the assignment of weights to six types of FI and the formulation of a new scoring system based on patients' and laypersons' perceptions were performed. In the second phase, the colorectal surgeons were also interviewed, and their assessment of the severity of different FI types was compared with the rankings of patients and laypersons.

First phase of the study: development of new scoring system

Study group: The first phase of the study comprised the patients and laypersons.

Patients group: (1) Inclusion criteria: the patients were defined as those who knew about FI and could potentially develop it secondary to the treatment of the disease condition they were suffering from. Therefore, patients suffering from anal fistula were included. Since they were experiencing the fear of incontinence, their perception was relevant; and (2) Exclusion criteria: the patients already suffering from any FI were excluded as they would rate their type of incontinence worse than other types.

Laypersons group: The layperson group was included because the patients with FI were expected to be emotionally involved. Therefore, a group that was not directly involved in the disease process was also included. Inclusion criteria: the relatives of the patients suffering from a disease, the treatment of which could potentially cause FI (anal fistula), were included. These persons were aware of the details and the risk of FI in their close relatives but were not actually directly afflicted by the disease. Therefore, they would balance out any extreme response by the patients.

Parameters of FI

The six parameters directly reflecting FI were included: solid, liquid, flatus (gas), urge, stress, and mucus.

Study methodology

Measuring weight for each FI type: The six FI parameters were defined in simple language (English as well as the native language, Hindi), which a patient and layperson could easily understand (Table 4). An interviewer was assigned to interview each person. Both the interviewer and interviewee (study subject) were blinded to the goals or the purpose of the study. The interviewees were shown the proforma, and after they understood the definitions of six FI parameters, they were asked to arrange the six parameters in order of decreasing severity. After this, each parameter was to be assigned numbers (disability score) between 0 and 100. The disability score was the measure of the impact of FI on all aspects of the life of the person. The worst parameter would be assigned a disability score of 100, and all other parameters would be assigned disability scores according to that. The same weight could be assigned to different parameters if the interviewee perceived them as of equal magnitude. In order to guide the interviewee regarding the weight assignment, a modified EQ-5D+ (EuroQol) description system was utilized[24]. EQ-5D+ includes a structured approach in which each health state parameter is described in terms of dimensions and severity levels within each dimension. In the present study, a 4D3L (4 dimensions, 3 levels) description system was used (Table 5). The impact of FI type on four dimensions of daily life, usual routine activity, anxiety/depression, self-esteem, and social life, was assessed, and a maximum of 25 points were assigned to each dimension. Each dimension had three levels, and depending on the severity level of that dimension, the points out of 25 were to be assigned to that dimension (Table 5). For example, an interviewee assessing liquid FI assigns 18 points to usual routine activity (out of 25), 20 points to anxiety/depression (out of 25), 15 points to self-esteem (out of 25), and 22 points to social life dimension (out of 25), then the total disability score assigned to liquid FI would be 75 (out of 100) for that interviewee (Table 5). Similarly, every interviewee would assign disability scores to each of the six types of fecal FIs. The 4D3L system was used to make the procedure objective and also helped the interviewee to fill the proforma easily. The two dimensions, self-esteem and anxiety, are relevant to evaluate the impact of a medical condition and were not given much importance in earlier scoring systems[25,26]. The inclusion of the parameters used as independent parameters in previous scoring systems like "Need to wear a pad"[17,18], "Alteration in lifestyle"[17,18], and "Need to take a constipating medicine"[18], were included as a part of the 4D3L proforma and therefore had an influence on assigning disability score to each type of FI. As discussed above, these parameters are not a type of incontinence, and their independent inclusion in the scoring system would have led to the error.

A pilot study was done before commencing the main study to assess any shortcomings. This was helpful in removing questions that were irrelevant or difficult to comprehend, improving the proforma's content, and making the language

Table 4 Detailed description of different types of incontinence [it was also translated into the local language (Hindi)]

Incontinence	Description
Urge	Whenever there is an urge to pass motion, normally, a person can hold the motion for a few minutes. In urge IC, the person faces difficulty holding the motions whenever there is an urge of passing motions. Although the motion does not come out, the feeling and fear that it will come out force the patient to rush to the toilet
Stress	Whenever there is an increase in pressure inside the tummy like in coughing or lifting weights, a person with normal anal sphincters can tighten his sphincters and hold the motions. A person can hold the motion for a few minutes. In stress IC, on increasing the pressure inside the tummy like in coughing or lifting weights, a little bit of motion or flatus leak from the anus
Mucus	A person with normal anal sphincters has a good anal tone due to which no leakage of mucus (normally present in the rectum as there is saliva in the mouth) occurs. However, in a person with weak sphincters, some mucus may leak out into the area around the anus spontaneously
Flatus	A person with normal anal sphincters has a good anal tone due to which he/she has control over the passage of gas/flatus. The person can hold the gas/flatus for some time. However, in a person with weak sphincters, gas/flatus may leak out of the anus with the person having no control over it
Liquid	A person with normal anal sphincters has a good anal tone due to which no leakage of liquid stool occurs. However, in a person with weak sphincters, some amount of liquid stool may leak out into the area around the anus spontaneously
Solid	A person with normal anal sphincters has a good anal tone due to which no leakage of solid stool occurs. However, in a person with weak sphincters, some amount of solid stool may leak out into the area around the anus spontaneously

Table 5 Four dimensions, 3 levels [modified EQ-5D+ (EuroQol)] description system utilized in the study and assignment of disability score for each fecal incontinence

Dimension	Dimension description	Perception of severity level
Usual routine activity	Performance of usual role activities such as working at a job, housework, child care, volunteer work, *etc.* Need to wear a pad, take a constipating medicine	Minimal problems with performing usual activities (0–5)
		Some problems with performing usual activities and moderate alteration in lifestyle (6–15)
		Unable to perform usual activities and severe alteration in lifestyle (16–25)
Anxiety/depression	Negative psychological states include anxiety, depression, behavioral, emotional control, loneliness, *etc.*	Minimal anxiety or depression (0–5)
		Moderate anxiety or depression (social isolation and loss of appetite) (6–15)
		Extremely anxious or depressed (suicidal ideation) (16–25)
Self-esteem	Perception about self	Minimal loss of self-esteem (0–5)
		Some loss of self-esteem (6–15)
		Marked loss of self-esteem (16–25)
Social life	How frequently the person goes out for socializing, like going to the cinema to watch a movie, going to a party, going out of the station for vacation	Minimal impact on social life (0–5)
		Some loss of social life (6–15)
		Marked curtailment of social life (16–25)

For example, an interviewee assessing liquid fecal incontinence assigns 18 points to Usual routine activity (out of 25), 20 points to anxiety/depression (out of 25), 15 points to self-esteem (out of 25), and 22 points to social life dimension (out of 25), then the total disability score assigned to liquid FI would be 75 (out of 100).

simpler. The subjects in the pilot study were not included in the final study.

Calculating the final weight for each FI type: The data of patients and laypersons were combined. The disability scores for each FI type were tabulated, and the average disability score for each FI type was calculated. Th weight for that FI was calculated by dividing the average disability score by 10 and rounding off the number to the nearest whole number (Table 6). This was done for simplicity, as a numbers with decimals would would have been difficult to use.

Development of a scoring system: Once the weights for each FI type were determined based on patients' and laypersons' perceptions, the three frequencies of each FI type were fixed as never (No episode of FI ever), occasional (≤ 1 episode of FI/ wk) or common (> 1 episode of FI/ wk). These were assigned linear scores of 0, 1 and 2, respectively. The frequency system of never, daily, weekly, monthly or yearly was not used for a few reasons. First, the patients found it difficult to calculate the frequency when there were too many columns. Secondly, the frequency of any FI type could vary in

Table 6 Weight assignment to different types of incontinence by study group

	Solid	Liquid	Flatus	Mucous	Stress	Urge
Patients' (n = 50) average disability score	82.5 ± 19.1	84.8 ± 15.4	58.1 ± 23.6	55.3 ± 21.2	52.0 ± 23.9	68.5 ± 23.5
Laypersons' (n = 50) average disability score	83.0 ± 22.4	81.4 ± 19.2	54.6 ± 21.1	55.2 ± 19.3	48.8 ± 22.0	68.3 ± 22.8
Total average disability score	82.7 ± 20.7	83.1 ± 17.4	56.3 ± 22.3	55.2 ± 20.1	50.4 ± 22.9	68.4 ± 23.0
Division by 10	8.27	8.31	5.63	5.52	5.04	6.84
Final weight (after rounding-off)	8	8	6	6	5	7

frequency over a period of time. Third, increasing the columns would have made the scoring system more cumbersome.

Second phase of the study: comparison of ranking of six types of FI according to severity by patients, laypersons and surgeons

In this phase, the assessment of FI was done by surgeons, and FI ranking in decreasing order of severity was done by three participating groups: patients, laypersons and surgeons. As discussed above, this was not done to assign weights or for formulation of the new scoring system but to check whether there was a correlation between patients, laypersons and colorectal surgeons regarding perception of severity of different types of FI.

Surgeons: Inclusion criteria: colorectal surgeons who had performed at least 30 anal fistula procedures in their surgical career were included. The persons in each group (patients, laypersons and surgeons) were asked to rank the six FI in decreasing order of severity. The most severe FI was given a rank of 6, and the least severe FI was given a rank of 1. The other types of FI were given ranks between 2 and 5, depending on the severity. The average of each FI type in each group was calculated, and the final ranking of FI in the groups was tabulated and compared (Table 7).

Ethics

The study was approved by the Ethics Committee of Adesh Medical College and Hospital, Shahbad, India (reference number AMCH/IEC/2022/02/04).

Statistical analysis

The categorical variables were compared by performing the χ^2 or Fisher's exact test. For normally distributed data, the continuous variables were tested by Student's t test when there were two samples, and analysis of variance (ANOVA) there were three or more samples. For non-normally distributed data, the Wilcoxon signed-rank test was performed for paired samples, and Mann–Whitney U test for unpaired samples. The significant cut-off point was set at $P < 0.05$.

RESULTS

Fifty patients and 50 laypersons were included in the first phase of the study. All the proformas were filled over a period of 1 year by the same interviewer between March 2022 and March 2023. The mean age was 40.7 ± 11.7 years (41.7 ± 12.0 in patients and 39.8 ± 11.4 in laypersons), and 71 were male (44 in patients and 27 in the laypersons groups).

The mean disability scores assigned to solid, liquid, flatus, mucous, stress, and urge FI by the patients were 82.5 ± 19.1, 84.8 ± 15.4, 58.1 ± 23.6, 55.3 ± 21.2, 52.0 ± 23.9, and 68.5 ± 23.5 respectively and by the laypersons were 83.0 ± 22.4, 81.4 ± 19.2, 54.6 ± 21.1, 55.2 ± 19.3, 48.8 ± 22.0, and 68.3 ± 22.8 respectively (Table 6). The overall mean disability scores assigned to solid, liquid, flatus, mucous, stress, and urge FI were 82.7 ± 20.7, 83.1 ± 17.4, 56.3 ± 22.3, 55.2 ± 20.1, 50.4 ± 22.9, and 68.4 ± 23.0 respectively (Table 6). After dividing these by 10 and rounding them off, the final weights assigned to solid, liquid, flatus, mucous, stress, and urge FI were 8, 8, 6, 6, 5, and 7, respectively (Table 6).

The three frequencies fixed for all FIs were never, occasional and common and were assigned points of 0, 1 and 2, respectively (Table 8). Thus, a new scoring system was finalized (Table 8). The maximum possible score was 80, which implied total incontinence, and the minimum score possible was 0, which implied normal continence (no incontinence) (Table 8).

In the second phase of the study, 50 patients and 50 laypersons from the first phase of the study were included, and 33 colorectal surgeons were interviewed to rank the six different types of FI according to severity. The ranking of the surgeons, patients and laypersons was compared. The perception of the patients and the laypersons correlated well, whereas it did not correlate with the surgeons' perception (Tables 7 and 9). The patients rated liquid FI, while the surgeons perceived solid FI as the most severe (Table 7). After solid and liquid FI, the patients and laypersons ranked urge FI as the most severe, whereas the surgeons ranked stress FI at that level (Table 7). The severity perception of the patients and laypersons regarding solid and urge FI was significantly different from the surgeons ($P < 0.00001$, ANOVA) (Table 9).

Table 7 Comparison of ranking of six types of fecal incontinence as per severity perceived by patients, laypersons and surgeons

Ranking	Patients (*n* = 50)		Laypersons (*n* = 50)		Surgeons (*n* = 33)	
Most severe to least severe	Type of FI	Ranking mean ± SD	Type of FI	Ranking mean ± SD	Type of FI	Ranking mean ± SD
6	Liquid	4.73 ± 1.25	Solid	4.80 ± 1.50	Solid	6.0 ± 0.0
5	Solid	4.51 ± 1.50	Liquid	4.64 ± 1.35	Liquid	5.0 ± 0.0
4	Urge	3.65 ± 1.52	Urge	3.70 ± 1.44	Stress	2.90 ± 1.07
3	Flatus	2.87 ± 1.50	Flatus	2.72 ± 1.45	Mucous	2.81 ± 0.91
2	Mucous	2.57 ± 1.38	Mucous	2.70 ± 1.44	Flatus	2.57 ± 1.06
1	Stress	2.53 ± 1.53	Stress	2.46 ± 1.38	Urge	1.69 ± 1.07

The persons in each group (patients, laypersons and surgeons) were asked to rank the six types of fecal incontinence (FI) in decreasing order of severity. The most severe was given 6 points, and the least severe FI was given 1 point. The average of each FI type in each group was calculated, and the final ranking of FI in that group was calculated.

Table 8 New scoring system

Incontinence type	Weight	Frequency			Maximum score
		Never (points)	Occasional (points) (≤ 1 episode/ wk)	Common (points) (> 1 episode/ wk)	
Solid	8	0	1	2	16
Liquid	8	0	1	2	16
Urge	7	0	1	2	14
Flatus	6	0	1	2	12
Mucus	6	0	1	2	12
Stress	5	0	1	2	10
Total					80

Score in a cell = Weight for that incontinence type × frequency points. For example, a person with occasional liquid incontinence would have an 8 × 1 = 8 score. Maximum possible score = 80 (total incontinence), minimum score possible = 0 (no incontinence).

Table 9 Difference in mean ranking six types of fecal incontinence as per severity perceived by patients, laypersons and surgeons

Type of FI	Ranking mean ± SD			Significance
	Patients (*n* = 50)	Laypersons (*n* = 50)	Surgeons (*n* = 33)	(ANOVA)
Solid	4.51 ± 1.50	4.80 ± 1.50	6.00 ± 0.00	$P < 0.00001$
Liquid	4.73 ± 1.25	4.64 ± 1.35	5.00 ± 0.00	$P = 0.35$
Urge	3.65 ± 1.52	3.70 ± 1.44	1.69 ± 1.07	$P < 0.00001$
Flatus	2.87 ± 1.50	2.72 ± 1.45	2.57 ± 1.06	$P = 0.88$
Mucous	2.57 ± 1.38	2.70 ± 1.44	2.81 ± 0.91	$P = 0.90$
Stress	2.53 ± 1.53	2.46 ± 1.38	2.90 ± 1.07	$P = 0.29$

The persons in each group (patients, laypersons, and surgeons) were asked to rank the six fecal incontinence (FI) in decreasing order of severity. The most severe was given 6, and the least severe FI was given 1 point. The average of each FI type in each group was calculated and compared.

DISCUSSION

The objective scoring of FI is an important and necessary tool, especially for gastroenterologists, gastrointestinal and colorectal surgeons, and neurologists. Out of the few scoring systems published, the Wexner and Vaizey scoring systems

are the most popular and commonly used[17,18]. However, as discussed above, there are a few major shortcomings in these scoring systems, due to which they are not accurate, comprehensive, and bias-free. In the present system, it was attempted to remove the lacunae of existing scoring systems (Table 10).

FI missed out in previous scoring systems, such as urge in Wexner, mucous in Wexner and Vaizey, and stress FI in all previous systems, were included in the new scoring system[18]. The new system is the first in which all six types of FI were included: solid, liquid, flatus, mucus, stress, and urge. It is logical to include all these six FI in a scoring system as all of these are distinct, and the presence of any of these indicates a malfunction in the coordinated function of a portion of the sphincter mechanism.

The biggest lacuna in the Wexner and Vaizey scores is that all types of FIs are assigned the same weight (Tables 1 and 2)[17,18]. In fact, no attempt was made to assign differential weights to different types of FI[18]. It is difficult to understand how all types of FI could be assigned exactly the same scores when all six FI types are different. The present study highlighted that weights calculated for different FIs could have significant variations ranging from 5 (urge FI) to 8 (liquid and solid FI) (Tables 6 and 8). Therefore, assigning these FIs equal weight was too simplistic and a source of significant error.

In the past, patients' perceptions were not given due importance, or it was assumed that surgeons' and patients' perceptions would be similar. We were of the opinion that the patients' subjective perception should be the basis of any FI scoring system. If there is any discrepancy between surgeons' and patients' perceptions regarding FI, then the patients' perceptions should be given priority over surgeons' perceptions. Even taking an average of both would be inappropriate. This is because, ultimately, the patients are the sufferers, and before making any decision regarding a treatment/surgery, fellow patients' perceptions would be of more relevance to any patient. Therefore, we did not include surgeons' perceptions in determining the weight of the different types of FI. In the second phase of the study, we compared the severity ranking of different FI according to the patients, laypersons, and surgeons' perceptions. The patients' and layperson's ranking of different FI was similar, but both were significantly different from the surgeons' rankings (Tables 7 and 9).

In the FISI scoring system, as in the present study, the surgeons' and patients' perceptions were different as the surgeons gave more importance to solid than liquid FI (Tables 3, 7, and 9). The reason mentioned in the FISI study was that surgeons viewed solid FI as a reflection of sphincter integrity and the adequacy of surgical repairs[19]. It was further discussed that professionals tended to assign a higher value to more severe elements, whereas patients placed greater importance on more common events[19,27]. In such a scenario where discrepancy arises, the patients' perception should be given priority in formulating a scoring system, and it would be inappropriate to force surgeons' opinions on the patients.

The FISI study also had several lacunae. When compared to the present study, the sample size in the FISI study was smaller (n = 34 *vs* 100 in the present study)[19]. About one-third of patients (12/34) in the FISI study completed the questionnaire through email[19]. Also, their questionnaire was not detailed and contained a single 20-cell table that was to be filled by the patients (Table 3)[19]. Each cell had to be assigned a number between 1 and 20 according to the severity, and no number could be repeated (Table 3)[19]. This step in their methodology could lead to error as two different cells could be perceived to be of similar severity by patients and could have deserved the same number (*e.g.*, mucus leakage once weekly could be perceived as severe as gas leakage once daily by the patient). Also, the present study showed that detailed definitions (Table 4) and the EuroQol descriptive system (Table 5) helped to guide the patients to understand the parameters properly and filling the charts accordingly. In our study, the interviewer took at least 1 h in every case to accurately fill out the chart. It seemed highly improbable that such detailed charts could be filled appropriately *via* email, as was done in the FISI study[19]. The proper statistical methods were also not followed in the FISI study, such as assigning weights to each FI type, linear increase in scores on the increasing frequency of symptoms, *etc.* Therefore, the end result of FISI scoring was haphazard and not amenable to usage (Table 3). This was perhaps the main reason that the FISI scoring system did not become popular and was not widely utilized. All the existing lacunae were removed in the present study, and the latter utilized detailed and sound scientific methods and in a much larger sample than in the FISI study (Table 10).

In the present study, like in the FISI study[19], the surgeons perceived solid FI as the worst, while the patients perceived liquid FI as the worst. Another interesting aspect was that after excluding solid and liquid FI, the surgeons perceived stress FI while patients and laypersons perceived urge FI as the worst (Table 7). On detailed questioning, most surgeons opined that in stress FI, there was actual leakage of fecal matter, while in urge FI, one had to rush to the toilet, and actual leakage could be prevented in most cases. In contrast, the patients and laypersons opined that stress FI would only happen when there was stress (lifting weights, and coughing, *etc.*), and many times, such stressful situations (and hence stress FI) could be avoided (not lifting weights) or curtailed (coughing lightly) but in urge FI, the person had to rush to the toilet every time, and this would hamper the quality of life in a bigger way. It was difficult to ascertain the reason behind this difference in perception between the surgeons and patients.

The parameters included in the existing scoring systems (Wexner and Vaizey) which were not a type of FI and were not a direct measure of the degree of FI like "Need to wear a pad"[17,18], "Alteration in lifestyle"[17,18], and "Need to take a constipating medicine"[18], which led to error, were excluded as parameters in the new scoring system. Rather, these were included in the 4D3L [modified EQ-5D+ (EuroQol)] description system (Table 5), which served as the basis for assigning weights to different types of FI. As discussed above, these parameters are the outcome/side effects of FI, and adding them as separate parameters for scoring was leading to a confounding bias.

It is pertinent that there is an optimal balance between scientific soundness and simplicity of a scoring system. Steps were taken to simplify the new scoring system, such as rounding off the assigned weights to the nearest whole number and decreasing the frequency of FI episodes from 5 (earlier scoring systems) (daily, weekly, monthly, yearly and never) to 3 (common, occasional and never). During the pilot study, it was realized that dividing the symptoms (episodes) into

Table 10 Comparison of existing scoring systems with new scoring system

	Wexner	Vaizey	FISI	NSS
Comprehensive	No	No	No	Yes
FI type included: urge FI	No	Yes	No	Yes
FI type included: mucous FI	No	No	Yes	Yes
Presence of confounding parameters like "Need to wear a pad", "Need to take constipating medicine", and "Alteration of lifestyle"	Yes	Yes	No	No
Assigning weights to each FI by an objective method	No	No	No	Yes
Inclusion of patient perceptions (*n*)	0	0	34	50
Inclusion of laypersons' perceptions (*n*)	0	0	0	50
Simple and easy to use	+++++	+++++	+	+++++
Detailed structured definitions	No	No	No	Yes
In-depth disability scores based on an objective description system	No	No	No	4D3L [modified EQ-5D+ (EuroQol)] used

FI: Fecal incontinence.

daily, weekly or monthly was difficult for the patients as the frequency of FI episodes is not strictly regular or periodic. Therefore, the broad categories of FI episodes as common or occasional made the scoring task easier for the patient while also simplifying the system without significantly impacting the scientific quotient. In contrast, giving equal weight to all types of FI for the sake of simplicity would be inappropriate as that would significantly compromise accuracy.

The subjective evaluation of study subjects could lead to deviation of the results. Therefore, several steps were taken to maximize objectivity while developing the new scoring system. First, in the proforma, the six FI parameters were defined in simple as well as two languages (English and the native language, Hindi), which a patient and layperson could easily understand. Second, both the interviewer and interviewee were blinded to the goals or purpose of the study. Third, all the proformas were filled out by the same interviewer. Fourth, to increase objectivity while filling out the proforma by the study subjects, a modified EQ-5D+ (EuroQol), 4D3L descriptive system was utilized. Scoring (0–25) was also utilized in each dimension to guide study subjects and to increase objectivity.

The study had some limitations. The new scoring system was not tested for inter- and intra-observer variability and test–retest reliability. However, these are planned for the next phase of the study. The validity of the new scoring system could not be checked because there was no gold standard against which it could be validated. The system was based on assigning weights and had more types of FI, due to which it was fundamentally different from the commonly used Wexner and Vaizey scoring systems. Nonetheless, this was a single-center study, and the new scoring system should be validated in a larger sample, preferably in multiple centers.

CONCLUSION

The new scoring system to objectively assess and grade FI clinically is more accurate than the existing scoring systems. It is simple and easy to use, and addresses most of the shortcomings of previous scoring systems. However, further studies are needed to corroborate the results of the present study.

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ARTICLE HIGHLIGHTS

Research background

Fecal incontinence (FI) is a common problem. Its assessment is difficult, and an objective scoring system needs to be developed so that FI can be evaluated uniformly all across the globe.

Research objectives

To develop a new effective, and scientifically sound scoring system in which the shortcomings of the existing scoring

systems are removed.

Research motivation
The existing scoring systems had many lacunae, due to which they were not scientifically accurate.

Research methods
A proforma was made in simple language in which all definitions of FI were included so that the study participants (patients and laypersons) could understand them. The study participants were then assigned disability scores (ranging from 0 to 100) for each type of FI based on a modified EQ-5D+ (EuroQol) descriptive system. The average score of each type of FI was calculated, divided by 10, and rounded off to determine the weight of each type. In the second phase of the study, a group of colorectal surgeons was asked to rank the six FI types in order of severity, and their ranking was compared with that of the patients and laypersons.

Research results
One hundred participants (50 patients and 50 laypersons) were included in the study. A new scoring system was formulated in which the maximum possible score was 80 (total incontinence), and the least 0 (no incontinence). The surgeons' ranking of FI severity differed significantly from the patients' and the laypersons' rankings, highlighting that the surgeons and the patients may perceive the severity of different FIs differently.

Research conclusions
A new scoring system for FI was formulated, which was simple, logical, comprehensive, and easy to use. The perceptions of patients and surgeons regarding the severity of different FIs differed significantly.

Research perspectives
The new scoring system would be useful for clinicians worldwide to objectively assess FI in the clinical setting.

FOOTNOTES

Author contributions: Garg P conceived and designed the study, collected and analyzed the data, revised the data, and finally approved and submitted the manuscript (Guarantor of the study); Sudoł-Szopińska I collected and analyzed the data, revised the data, finally approved and submitted the manuscript; Kołodziejczak M critically analyzed the data, reviewed and edited the manuscript, finally approved and submitted the manuscript; Bhattacharya K analyzed the data, revised the data, finally approved and submitted the manuscript; Kaur G analyzed the data, revised the data, and finally approved and submitted the manuscript.

Institutional review board statement: The study was approved by the Ethics Committee of Adesh Medical College and Hospital (Approval No. AMCH/IEC/2022/02/04).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at drgargpankaj@gmail.com.

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