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ABOUT COVER

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REVIEW

Current understanding of the impact of COVID-19 on gastrointestinal disease: Challenges and openings

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Abstract

The novel coronavirus disease-2019 (COVID-19) is caused by a positive-sense single-stranded RNA virus which belongs to the Coronaviridae family. In March 2019 the World Health Organization declared that COVID-19 was a pandemic. COVID-19 patients typically have a fever, dry cough, dyspnea, fatigue, and anosmia. Some patients also report gastrointestinal (GI) symptoms, including diarrhea, nausea, vomiting, and abdominal pain, as well as liver enzyme abnormalities. Surprisingly, many studies have found severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral RNA in rectal swabs and stool specimens of asymptomatic COVID-19 patients. In addition, viral receptor angiotensin-converting enzyme 2 and transmembrane protease serine-type 2, were also found to be highly expressed in gastrointestinal epithelial cells of the intestinal mucosa. Furthermore, SARS-CoV-2 can dynamically infect and replicate in both GI and liver cells. Taken together these results indicate that the GI tract is a potential target of SARS-CoV-2. Therefore, the present review summarizes the vital information available to date on COVID-19 and its impact on GI aspects.

Key Words: SARS-CoV-2; COVID-19; Gastrointestinal symptoms; Recommendation; Diagnosis; Therapeutics

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Core Tip: The landscape of coronavirus disease-2019 (COVID-19) is evolving dramatically, with new information increasing at an alarming rate. It is a challenge to make sense of these data and to interpret what is crucial and high-quality evidence. In this critical circumstance, in-depth work is highly important for the future treatment and management of the disease. In this review, we summarize the vital information available to date on COVID-19 and its impact on gastrointestinal aspects.

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INTRODUCTION

Populations worldwide are currently facing an unprecedented health emergency due to the spread of novel coronavirus disease-2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). According to the World Health Organization (WHO), the COVID-19 health crisis has spread to over 205 countries including the United States, India, Russia, Brazil, and Colombia[1-3]. The most common symptoms of moderate COVID-19 include fever, dry cough, tiredness, sore throat or dyspnea. However, the pulmonary system is the main system involved in the clinical manifestation of the disease. Patients infected with this virus can suffer potential damage to other vital organs, such as the gastrointestinal (GI), cardiac, renal and nervous systems[4-6]. Before COVID-19, many infectious diseases affected global populations, including the plague, Spanish flu, cholera, swine flu (H1N1), and severe acute respiratory syndrome-coronavirus (SARS-CoV)[7-11]. Recent studies have shown that the mortality ratio (MR) due to COVID-19 in China is 0.66%, on the Diamond Princess ship it is 2.3% and a large meta-analysis of 36 European countries showed that the MR ranged from 4% to 4.5%[12-14].

To date, there are no vaccines or medicines available to combat this pandemic. However, several clinical trials of both therapeutics and vaccine candidates are underway. Furthermore, it was proven that convalescent plasma transfusion (CPT) is useful in patients with severe COVID-19[15,16]. As the landscape of COVID-19 is evolving dramatically, it is becoming a challenge to determine high-quality evidence as data is being generated at an alarming rate.

Several studies have identified SARS-CoV-2 viral RNA in rectal swabs[17,18], and stool specimens of asymptomatic COVID-19 patients[19,20]. This raises the issue of GI viral infection and the route of fecal-oral transmission. Furthermore, viral receptor angiotensin-converting enzyme 2 (ACE2) is expressed in epithelial cells of GI mucosa[21,22]. Taken together, these results indicate that SARS-CoV-2 can dynamically infect and replicate in the GI tract and liver cells. However, this has important implications for the management, transmission and control of the disease. Thus, in-depth research is essential for the future treatment and management of the disease. In this review, we summarized the vital information available to date on COVID-19 and its impact on GI aspects. We recognize the ever-changing literature and aim to update future publications with the most up-to-date information available.

GI MANIFESTATIONS OF COVID-19

There are more than 40 million cases of COVID-19 worldwide, and studies that can confirm the symptoms and manifestations associated with SARS-CoV-2 are now critical. Although the lung is the primary target organ of this virus, this causes discomfort in patients with detrimental oxygen saturation effects, causing shortness of breath. However, GI symptoms have also been observed in COVID patients[23-25]. If the virus enters *via* food it may cause diarrhea. In patients with high viral load, anorexia, anosmia, and dyspepsia are the main GI symptoms[26,27]. Furthermore, anti-viral drugs can give rise to nausea, vomiting and diarrhea (Table 1).

Table 1 Gastrointestinal manifestations of coronavirus disease-2019

Gastrointestinal manifestations	Clinical findings
Lack of appetite	Elevated AST
Anorexia	Elevated ALT
Anosmia	Elevated bilirubin
Vomiting	Elevated LDH
Dysgeusia	
Nausea	
Abdominal pain	
Bloody diarrhea	
Intestinal dysfunction	

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase.

SARS-CoV-2 can be detected in the GI tract. The virus can invade epithelial cells in the stomach, intestine and colon surface and cause symptoms in the GI tract. As the world is now facing the second wave of this current pandemic, the GI manifestations of COVID-19 require much more attention as there are more possibilities for transmission of the infection due to lack of information/knowledge on GI complications[21,28-30]. The course of alveolar events, GI spread and the manifestations of SARS-CoV-2 infection are shown in Figure 1.

A meta-analysis of 60 studies from 6 countries found that 4243 patients with SARS-CoV-2 infection had GI symptoms, including diarrhea (12.5%), nausea/vomiting (10.2%), anorexia (26.8%) and abdominal pain (9.2%), which were the most common symptoms[31]. Another hospital-based study reported that of 1099 patients, nausea or vomiting and diarrhea occurred in 5% and 3.8% of patients, respectively[21]. Another study showed that in 148 Chinese SARS-CoV-2 positive patients, half of the patients seemed to have abnormal liver function at the time of admission[32]. A further study showed similar results with non-typical findings in hepatic enzymes[33,34]. Patients were expected to have moderate to high fever with elevated liver function, and these tests were remarkably more frequent in male patients (68.67%) than in female patients (38.36%)[35].

CLINICAL SIGNIFICANCE OF COVID-19

SARS-CoV-2 when entering the body causes viremia with associated pneumonia and the main clinical features of this disease are fever, severe headache, fatigue, diarrhea and other potential associated comorbidities[36,37]. The estimated average duration of incubation is 1-14 d (commonly 3-7 d). Viruses enter the body and proliferate mainly in the lungs, GI tract, and heart. SARS-CoV-2 is believed to be concentrated in tissues that express ACE2[38,39]. Recently, studies have reported that anorexia, nausea, vomiting and diarrhea were the most common GI tract symptoms in almost 40% of patients[40,41]. However, one-tenth of patients complained of GI symptoms without major difficulty in breathing or mild fever[42]. SARS-CoV-2 has been correlated with a hyper-coagulable condition with a major risk of venous thromboembolism[43]. Neurological indications such as severe headache, dizziness, and loss of consciousness, stroke, and muscle injury have also been observed[44]. Detailed clinical manifestations are shown in Table 2. GI manifestations of SARS-CoV-2 infection are also indicated to alter nutrient absorption by modulating the expression and activity of nutrient transporters, such as the neutral amino acid transporter BAT1.SARS-CoV-2 infection which potentially triggers physiological and anatomical damage related to the GI tract along with its systemic influence is summarized in Figure 2.

Table 2 Clinical significance of coronavirus disease-2019

Stage	Symptoms
Mild	Initial symptoms are mild or negligible with no sign of pneumonia on imaging.
Moderate	Cough, moderate fever, myalgia, gastrointestinal symptoms, anosmia and respiratory signs with radiological imaging findings of pneumonia.
Severe	The presence of one of the following: (1) Shortness of breath (RR ≥ 30 breaths/min); (2) Oxygen saturation ≤ 93% at rest; (3) Arterial partial pressure of oxygen/fraction of inspired oxygen ≤ 300 mmHg (1 mmHg = 0.133 kPa); and (4) In less than 24-48 h, more than half of patients with radiological imaging show clear lesion progression.
Critical	Any of the following: (1) Lung failure or requiring mechanical ventilation; (2) Septic shock; and (3) Multiple organ failure (other organ failure that requires HDU/ICU critical care.)

RR: Risk ratio; HDU: High dependency unit; ICU: Intensive care unit.

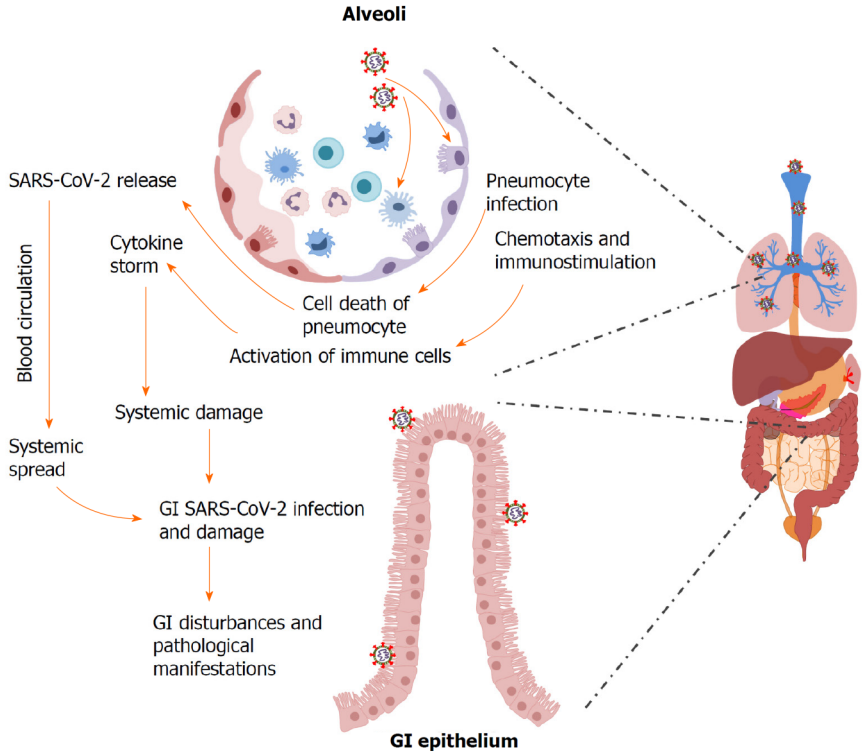

Figure 1 Systematic representation of the course of alveolar events, gastrointestinal spread and manifestations of severe acute respiratory syndrome coronavirus 2 infection. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; GI: Gastrointestinal.

CARE OF PATIENTS WITH EXISTING LIVER DISEASE

Non-alcoholic fatty liver

Obesity is a major risk factor for severe COVID-19 because pneumonia is mainly exacerbated in obese people as adipose tissue can act as a viral repository and is, therefore, an immunological center for the inflammatory response[45,46]. Since non-alcoholic fatty liver disease (NAFLD) is associated with metabolic comorbidities such as type 2 diabetes, hypertension and obesity, NAFLD patients are at higher risk of having severe COVID-19[47]. According to a retrospective study, 39 of 202 confirmed COVID-19 patients had higher body mass index (BMI) and higher NAFLD co-morbidity rates. The study also found that NAFLD patients had a higher risk of disease progression to severe COVID-19, and these patients also had a longer duration of viral shedding[48]. Interestingly, patients with NAFLD and SARS-CoV-2 infection less than 60 years of age are associated with an elevated risk of severe disease[49]. NAFLD patients with increased non-invasive liver fibrosis scores are more likely, despite metabolic comorbidities, to develop severe COVID-19 disease[50].

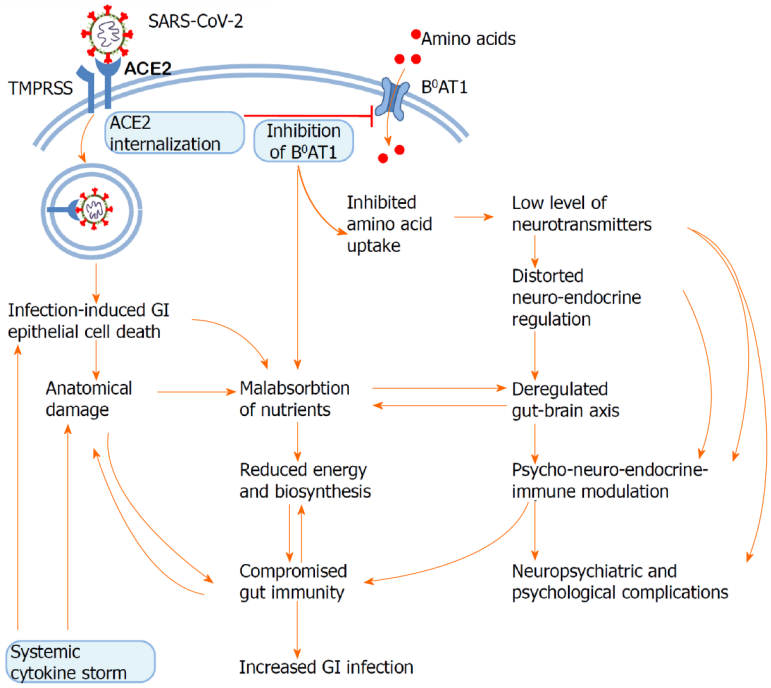

Figure 2 Potential events caused by severe acute respiratory syndrome coronavirus 2 infection in gastrointestinal physiological and anatomical damage with systemic influence. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE2: Angiotensin-converting enzyme 2; GI: Gastrointestinal.

Viral hepatitis

Almost 5 billion people in the Asia-Pacific region have chronic viral hepatitis[51]. Unlike metabolic liver disorder, there is little or no evidence that chronic viral hepatitis affects the progression of COVID-19. Corticosteroid and tocilizumab are administered to critically ill patients with COVID-19, which can lead to reactivation of hepatitis B virus (HBV) and to severe liver failure in patients with long-term HBV infection. Therefore, HBsAg screening should be performed in patients with severe COVID-19 with a positive HBsAg test at the time of corticosteroid therapy[52]. It is essential to initiate antiviral medication in newly diagnosed HBV patients affected by COVID-19 when HBV DNA is more than 2000 IU/mL and alanine aminotransferase is above the upper limit of normal. Drug-drug interactions between agents used to treat COVID-19 and HBV may occur. In addition, patients with HBV and HBC should continue to receive antiviral drugs. Considering the uncertain effect of interferon-alpha on the systemic inflammation associated with COVID-19, treatment with alternative drugs in patients with HBV during the pandemic should be addressed prior to medication administration. In COVID-19 patients, initiation of treatment for HBV and hepatitis C virus (HCV) is usually not required and should be delayed until recovery from COVID-19 is achieved. Interactions between the drugs used in the treatment of COVID 19, and those used to treat HBV and HCV should be carefully monitored.

Autoimmune liver disease

Patients with autoimmune liver disease can experience severe liver problems. Therefore, the reduction of immunosuppressive drugs in the prevention of SARS-CoV-2 is generally not recommended. The reduction should only be performed under special conditions (such as drug-induced cytopenia or bacterial super-infection) after consultation with a specialist. Treatment with corticosteroids was found to be promising in hospital patients seeking respiratory support for COVID-19. There is still concern that patients who are taking elevated doses of corticosteroids may be more vulnerable to SARS-CoV-2 infection and serious COVID-19. Patients who are already taking corticosteroids when they develop COVID-19 should have an adequate dose of corticosteroids to prevent adrenal insufficiency. Adding or switching to dexamethasone can only be addressed in patients with COVID-19 who require hospitalization and respiratory assistance. Liver enzymes such as transaminases, alkaline phosphatase and gamma-glutamyltranspeptidase may be increased in patients with confirmed COVID-19 or suspected SARS-CoV-2 infection. Thus, it is

strongly recommended that suspected infection should be confirmed by biopsy. However, if there is a strong suspicion of autoimmune hepatitis, empiric therapy may be initiated without histological confirmation at the standard treatment dose[53-55].

Liver cirrhosis

Patients with cirrhosis are susceptible to SARS-CoV-2 infection, new and worsening hepatic decompensation, severe COVID-19 and death. Patients with new or deteriorating hepatic decompensation should be given priority to SARS-CoV-2 screening even in the absence of respiratory symptoms. For patients with cirrhosis who are infected with SARS-CoV-2, rapid admission must be considered to prevent further worsening of their condition. There is no specific consideration to date for cirrhosis patients who have been infected with SARS-CoV-2 as the drug used to control COVID-19 may increase the risk of other infections and viral shedding[53,56,57].

Liver transplant recipients

Patients who have undergone liver transplantation are at high risk of severe infection with SARS-CoV-2 and death. The risk of transmission of SARS-CoV-2 through liver transplantation is unknown; therefore, it is recommended that all donors should be screened for SARS-CoV-2 infection with real-time reverse transcription polymerase chain reaction (RT-PCR). Considering that SARS-CoV-2 can spread from asymptomatic people, including children, patients undergoing liver transplantation should strictly follow physical distancing or not travel during the COVID-19 pandemic. Telehealth home surveillance is efficient and useful for liver transplant recipients; consequently, it must be available in most transplant centers and applied to pre-transplant patients where telehealth services are available. Unrecognized COVID-19 significantly increases the risk of severe immune suppression and post-transplant infection in liver transplant recipients, leading to multiple organ failure and even death. A reduction of immunosuppressive therapy in liver transplant recipients should not be considered to prevent SARS-CoV-2 infection. Such a reduction can only be carried out under exceptional conditions (including drug-induced lymphopenia or bacterial/fungal super-infection in the event of SARS-CoV-2 infection) after consultation with a specialist. Calcineurin inhibitor dosage levels and mechanistic targeting by rapamycin inhibitors should be closely monitored as they are offered in conjunction with drugs such as hydroxychloroquine, protease inhibitors or recent COVID-19 test drugs[58-61].

DIAGNOSIS OF GI COVID-19 EFFECTS

COVID-19 is spread by air-borne viral particles and affects not only the respiratory tract, cardiovascular, and central nervous systems but also affects the GI system. Previous findings demonstrated that several patients with COVID-19 had many low to moderate GI complications, including diarrhea during the disease course[26]. Until recently, there was no evidence on the potential of anti-GI drugs but sufficient frequent rehydration and potassium ion monitoring were conducted in COVID-19 patients. Therefore, it might be hypothesized that diarrhea should be considered an awareness parameter and must be investigated to reach an early diagnosis in COVID-19 patients. Furthermore, measurement of calprotectin could play an important role in the monitoring, diagnosis, and follow-up of COVID-19-associated diarrhea and GI complications. Therefore, participation of the GI tract in COVID-19 should be considered and explored under several clinical policies and practices such as the incorporation of rectal swab testing before the discharge of COVID-19 patients[62], as well as our future supply of personal protective equipment (PPE) in the endoscopy, ultrasound and another relevant diagnostic settings. These implementations will act as promising tools to eradicate COVID-19[63].

GI endoscopy

Endoscopy is a very complex procedure in GI clinical settings. In COVID-19 patients, it is a high-risk process for healthcare professionals due to potential high exposure while performing upper GI endoscopy, and makes them more prone to infection as a result of the patient's aerosol[64]. A study reported the presence of SARS-CoV-2 RNA in a patient's stool samples. These findings raise suspicion and support the potential fecal-oral transmission route of infection. Furthermore, the novel virus is possibly transmitted through fecal contamination *via* inhalation, conjunctival splash contact, or direct contact with feces during colonoscopy[21]. To restrict the aerosol transmission of

SARS-CoV-2 within/between the endoscopy team, various hospital infection control guidelines and protocols should be established. All endoscopic procedures should be conducted in an isolated aseptic environment with one set of endoscopy equipment and a PPE kit. According to recent data, it was estimated that the half-life of SARS-CoV-2 virus particles in the air is estimated at around 1.1 h[65]. To prevent viral load, patient to patient cross-infection, and transmission, the current guidelines suggest performing endoscopy in a negative pressure isolated room[66]. All COVID-19 patients should undergo prescreening before arrival at the endoscopy room. During this current outbreak of COVID-19, many experts have recommended the indications for urgent endoscopy which are limited to acute GI bleeding, GI obstruction requiring stenting or dilatation, biliary sepsis, repair of GI perforations and leakage, foreign body retrieval, and the establishment of enteral nutrition. According to recent guidelines, we would like to recommend other criteria including a minimum number of staff with specifically one proficient endoscopist and two nurses for each endoscopy platform. Adequate time should be allowed for infection control measures before and after endoscopy. It should be kept in mind that the same staff should serve the same room for the whole session while performing the procedure. However, when it comes to operative and non-operative endoscopic procedures, all safety measures concerning medical health worker exposure should be considered. There are three criteria and routes for the endoscopic procedure: (1) Oral route: any procedure *via* the mouth or nose; (2) Anal or stomal route: any procedure passing through the anus or an entero-cutaneous stoma; and (3) Capsule endoscopy.

The following vital parameters should be documented before performing endoscopy; fever of more than 37.5°C, contact history, travel history, occupational exposure, and clustering[67]. For all suspected and positive cases, the clinical preparation for endoscopy should be reviewed, and preferably patients with a medical emergency, life-threatening conditions should receive endoscopy. Moreover, all endoscopy teams and staff should receive proper training on the use of PPE and infection control management. Various disinfectants such as ethanol (62%-71% concentration), 2% glutaraldehyde, and 0.1%-0.5% sodium hypochlorite are frequently used before and after each case. All these practices can help to reduce the viral load within one minute of the exposure period[68].

Endoscopy is a very advanced tool, and we would like to recommend all elective endoscopies during the current COVID-19 pandemic for diagnosis purposes be limited until there is a promising cure. This diagnostic strategy helps to protect health care capacity to handle many suspected and positive cases of COVID-19 strategically. However, this approach will act as a potential protective measure to reduce the cross-transmission of COVID-19 between patients and healthcare staff, particularly at the early stage of the COVID-19 pandemic[69]. All these measures aid some significant effort to reduce the risk of cross-infection, the spread of the virus and preserve the use of PPE, are essential in overcoming the spread of COVID-19 within healthcare staff.

Abdominal ultrasound and computed tomography imaging

In COVID-19 patients, fever and dry cough are the most commonly observed symptoms? RT-PCR is the most frequently use confirmatory test to diagnose and identify genomic RNA of SARS-CoV-2. However, early radiological studies focused on imaging of the chest using high resolution computed tomography (CT), which measures peripheral patches of ground-glass densities with or without consolidations with bilateral basal predominance, organizing pneumonia pattern, crazy paving, mild bronchiectasis, and vascular engorgement may also be observed[70,71]. Radiography of the chest, apart from its widespread use and low cost, also has low diagnostic sensitivity for pneumonia in COVID-19 patients[72]. Unlike radiography, lung CT is considered a sensitive imaging technique for early detection of pulmonary severity in infected patients[73]. A recent study found that CT imaging is superior to RT-PCR (98% *vs* 71%, respectively, $P < 0.001$) in the diagnosis of COVID-19[74]. However, it is difficult to imagine a situation where CT is systematically performed in all suspected and positive cases due to cost, exposure to radiation, time required, and the probability of cross-contamination.

Ultrasound (US) is a portable, low cost, and relatively fast procedure with real-time visualization. In the current pandemic, US is considered a potent technique for the diagnosis of suspected and confirmed COVID-19 cases to facilitate better treatment strategies. The use of US has advantages as it restricts exposure of multiple staff to confirmed cases. Additionally, point-of-care US has recently been recommended to manage COVID-19 cases to reduce the use of medical equipment and the number of healthcare staff, which ultimately minimizes cross-infection. More recently, some authors have reported the high diagnostic accuracy of US compared with radiography

when evaluating lung abnormalities[75,76]. However, imaging findings may also increase the understanding of abdominal manifestations in COVID-19 patients. Recently, Abdelmohsen and coworkers conducted abdominal imaging studies (sonographic examination) at 30 intensive care units with 41 confirmed COVID-19 patients with abdominal complications. They reported that 51.2% of patients had increased liver function tests, particularly serum bilirubin, followed by elevated renal function tests in 14.6% of patients[77]. Tullie *et al*[78] conducted abdominal US and CT imaging in pediatric COVID-19 confirmed patients and found that US was the best diagnostic imaging tool in patients with GI symptoms.

Furthermore, they reported that US findings were in line with lymphadenopathy and the presence of inflammatory fat throughout the mesentery with visible thickening of the terminal ileum. Thus, US and CT may accelerate the understanding of abdominal complications in COVID-19 patients[78]. Therefore, we strongly recommend the use of abdominal US imaging for the diagnosis of patients with GI manifestations when investigating for possible appendicitis.

RISK FACTORS DURING ENDOSCOPY IN COVID-19 PATIENTS

Endoscopy is a minimally-invasive technique and is usually recommended for the evaluation of ulcers/polyps, gastritis, stomach pain, dysphagia, digestive tract bleeding, and sometimes for biopsy[79]. It is a safe and common practice; however, it does have the risk of adverse events preventing completion of the procedure and can result in complications ranging from low to potentially rare or mild to lethal complications[80]. During clinical practice from pre-procedure to post-procedure, various adverse reactions can occur, such as infections, cardiopulmonary disorders, bleeding, thromboembolism *etc.* To date, more than 44 million COVID-19 cases with over 1 million deaths have been recorded[81]. It has been verified that this infection is contagious and is chiefly transmitted *via* respiratory droplets (> 5 to 10 μm in diameter) or by close contact (usually within 1 m) to an infected individual while coughing or sneezing. The alarming element concerning COVID-19 is its airborne transmission that may also be feasible in some medical circumstances. Certain clinical practices such as endotracheal intubation, bronchoscopy, nebulization *etc*, produces aerosols resulting in aerial spread, while fecal-oral route transmission (fomites) can also result in contamination[82,83].

Paramasivam and his team conducted a review referencing the report of the Quality Committee of American Society for Gastrointestinal Endoscopy where they mentioned the three key rudiments in defining endoscopic risk factors, *i.e.*, the complexity of the procedure (procedure-related), co-morbidity and clinical status (patient-related) and individual expertise (operator-related)[84,85]. Complexity encompasses clinical and perceived risk elements. Technically more sophisticated approaches are often riskier. Treatment with endoscopic retrograde cholangiopancreatography (ERCP) in conjunction with or without sphincter division (sphincterotomy), colonic polyps as well as peptic ulcer elimination, have high complication rates following therapeutic endoscopy procedures[86]. Artificial ventilation or positive insufflation practice may cause significant aerosol generation due to short physical distancing during endoscopy, increasing the risk of infection in staff, medical personnel, anesthesiologist and others[87,88]. Techniques have progressed to endo-luminal from surgical methods with the escalation and advancement of endoscopic expertise, innovation and has accrued considerable experience to combat this pandemic[86,87].

Although, different endoscopic procedures have specific complications, such as post-ERCP pancreatitis in ERCP or asthma and hemoptysis in bronchoscopy[86,89]; however, perforation, hypoxemia and bleeding are the most commonly recorded complications of all forms of endoscopy[86]. It has been documented that the bleeding rate ranges from low to high with the intensification of invasive procedures such as endoscopic biliary sphincterotomy, endoscopy dilatation or colonoscopic polypectomy during diagnosis[90-92]. Medical guidelines recommend that endoscopy is carried out within 24 h of the patient presenting with acute upper GI bleeding[93]. Compared to other risks, endoscopic-mediated bleeding, typically improves after rapid and rigorous treatment and controls or minimizes other risks[90]. However, the debate about endoscopy in COVID-19 patients, gives rise to specific management decisions. A case study by Cavaliere *et al*[88] documented that endotracheal tube intubation during upper endoscopy surgery in a COVID-19 patient is challenging, and may increase the mortality rate. Coagulopathy linked to COVID is another explanation for GI bleeding; therefore, for stratification, prothrombin time, platelet count and D-dimer

measurement are recommended[94]. Since the complications of endoscopy could surpass the benefits, clinicians have agreed to cautiously treat these patients with a blood transfusion (when necessary), proton pump inhibitor drip, and regular surveillance of GI symptoms, hemoglobin level and other vital signs[88].

Similar to bleeding, perforation has also been recognized as an important risk factor due to its high incidence during numerous procedures, it has been studied in GI trials and clinical interventions are often employed[80]. Perforations are frequently found in the sigmoid colon. Three approaches are considered to cause perforations: air insufflation driven barotrauma, therapeutic procedures and colonoscopy or an instrument triggered mechanical injury[95,96]. Perforations occurring during ERCP arise when there is a transmural extension of sphincterotomy beyond the sphincter and are archetypally asymptomatic. Other factors include adhesions, dysfunction of the sphincter of Oddi, biliary stricture dilation, snare polypectomy *etc*[80].

Operator inexperience has also been designated a significant risk factor. Researchers found that prolonged treatment in conjunction with elevated sedation dose could be attributed, in part, to a higher proportion of complications. Over-sedation may increase the risk of severe complications (such as perforation and bleeding), which can inhibit the pain response[97,98]. Due to its short recovery duration, propofol is among the most widely used agents and has lower complication rates than conventional sedative agents as its mean sedation period is shorter and the sedation depth is higher. Propofol is thought to be safe for sedating (senior) patients when given the correct dose, while the risks including hemodynamic and breathing depression occur and accelerate during upper GI endoscopic procedures[80]. Under severe sedation, patients undergoing difficult colonoscopy tend to vomit resulting in pulmonary aspiration. This can lead to needless misperception in medical personnel and caretakers as the signs of pneumonia due to pulmonary aspiration are similar to those in COVID-19[99].

Respiratory and cardiovascular complications have also been observed. Tachycardia and bradycardia can be found during invasive events. Hypertension, hypotension, and syncope were observed during treatment. Clinical micro-aspiration can be considered insignificant if it does not result in pulmonary inflammation or prolonged bronchospasm[80]. As soon as these risk factors have been identified and defined, standard operating procedures and detailed protocols can be set up to reduce the risk of complications before or during the planning phase. The compulsory pre-screening test should be carried out before entry (conditionally carried out to the outbreak of COVID-19) and there should be a discrete unit for 'low-risk' and 'high-risk' patients. Also, healthcare staff and workers must abide by asymptomatic carrier precautions and level 2 biosafety[100].

RECOMMENDATIONS FOR THE MANAGEMENT OF PATIENTS WITH LIVER DISEASE DURING COVID-19

General recommendations

In view of the significant multifaceted effects of the pandemic, especially in chronic diseases, liver damage can be highly variable and complex, leading to the activation of an intra-hepatic immune response, triggering microvascular thrombosis, hepatic obstruction and systemic inflammation in addition to drug toxicity. In conjunction, this systemic disorder is often associated with a phenomenon known as "bystander hepatitis" and the patient can have a lethal course and show no specific signs of hepatic failure[101,102]. The personal care of these patients relies greatly on the regional COVID-19 prevalence and the laws and guidelines imposed[101]. Therefore, the pragmatic structuring of strategies to address this issue must be made by physicians and their organizations by strengthening electronic health records (EHR) and encompassing novel technologies such as remote monitoring and telemedicine to restore treatment levels wherever feasible[103]. The epidemiology of this virus appears uncertain. However, for some time to come, its prevalence may increase and diminish chronologically. Thus, a customized and versatile patient care strategy is needed to align nationwide SARS-CoV-2 infection dynamics, public infrastructure accessibility, and the degree of frequency of existing hepatic disease in an individual. Finally, it is necessary to restart clinical trial registration to make significant progress despite unimaginable global events[53] (Table 3) .

Standard recommendations

Both the American Association for the Study of Liver Diseases[104] and the European

Table 3 Recommendation guidelines for the management of patients with liver disease during coronavirus disease-2019

S. No.	Clinical condition	Consulting organization	Recommendation guidelines for management during COVID-19
1	Out-patient care	AASLD, EASL-ESCMID	(1) Offering telehealth; (2) Mail order of prescriptions & medications; (3) If viral hepatitis occurs: continue medication; (4) Tracking & recording alcohol usage; (5) Limiting testing, imaging & blood withdrawal; and (6) For patients with autoimmune liver disease, immunosuppression medication is continued.
2	In-patient care	AASLD, EASL-ESCMID	(1) Clustering COVID-19 & non-COVID-19 patients separately; (2) Minimizing personnel on rounds; (3) Safe discharge planning; (4) Usage of remote care-telehealth communications & video monitoring; (5) Limiting patient visitors; (6) Minimizing testing, imaging & blood withdrawal; and (7) Avoiding inter/intra-transfer between facilities.
3	Endoscopy	AASLD, EASL, APSDE, AGA, ESGE, ASGE	(1) Limiting emergent indications such as ERCP (for cholangitis), severe GI bleeding or variceal bleeding; (2) Minimizing personnel during procedures; (3) Every clinician/personnel recommended to use N95 masks and PPE as there is high aerosol generation during clinical procedures; and (4) Postponing certain elective procedures such as esophageal variceal screening.
4	NAFLD	AASLD, EASL	(1) Notification to patients regarding adverse hepatic/metabolic implications associated with social isolation & lifestyle; (2) In line with existing directives, arterial hypertension treatment should continue; and (3) All NAFLD patients who may be infected with SARS-CoV-2 should have early admission.
5	Viral hepatitis (HBV & HCV)	AASLD, EASL	(1) If under care, continue treatment for chronic HCV and chronic HBV; (2) For follow-up patients, offer telehealth and laboratory testing; (3) Mail order of direct-acting anti-viral prescriptions & medications, if initiated; (4) Alternative therapy should be considered as associated risks of IFN-α is unknown; (5) Case-by-case basis decision in consultation with a medical specialist should be undertaken for patients with COVID-19 and high disease flare; and (6) Use of anti-viral therapy is considered in individuals with resolved or chronic HBV and COVID-19 conditions undertaking immunosuppressive therapy.
6	Liver cirrhosis	AASLD, EASL	(1) Clustering COVID-19 & non-COVID-19 patients separately; (2) Early admission and prioritized COVID-19 testing for patients with ACLF or deteriorating/chronic hepatic conditions is advised; (3) Every attempt must be made, wherever feasible, to restore highest quality treatment for patients; (4) Prophylactic course of action for GI hemorrhage, hepatic encephalopathy *etc*. must be trialed; (5) Use of vasoconstrictor therapy ought to be undertaken with great consideration and care; and (6) Vaccination recommended for *Streptococcus pneumoniae* and influenza.
7	ALD	AASLD, EASL	(1) It is recommended that there should be no reduction in immunosuppressant dosing in patients with ALD & COVID-19. Under special conditions, dosage may be decreased but, after consultation with a clinician; (2) Monitoring of corticosteroid treatment in patients with elevated doses as they have increase susceptibility to viral infection; (3) Agents such as budesonide is recommended as a primary treatment to reduce the systemic risk of glucocorticoids; and (4) Vaccination is recommended for *Streptococcus pneumoniae* and influenza.
8	ARLD	AASLD, EASL	(1) Reduction in consumption of alcohol; (2) Implementing strategies such as cessation and online (telephone) alcohol liaison services; (3) Monitoring of corticosteroid treatment in patients with elevated doses as they have an increase susceptibility to viral infection; (4) Awareness of online circulation of misinformation or fabrication concerning alcoholic effects.
9	Liver transplantation and surgery	AASLD, EASL, ILTS, LTSI, ATS, TTS	(1) Avoid evaluation of in-patient transplants; (2) Screening of recipients and donors for COVID-19; (3) Reduction in immunosuppression in chronic COVID patients; (4) Routine reduction in immunosuppression doses should not be encouraged; (5) Edge to urgent indications/case-by-case; (6) Minimize workforce during treatment procedures; (7) Safe anesthesia practice with appropriate PPE and N95 masks use is recommended; and (8) Deferring elective procedures such as hepatic resection.
10	Hepatocellular carcinoma	AASLD, EASL, ILCA, ASCO, ESMO	(1) Postponing HCC screening for some months; (2) Pausing enrolment in clinical trials; (3) If surgery or extirpation are delayed, then trans-arterial bridging therapies should be offered; and (4) The patient needs to continue, if already taking tyrosine kinase inhibitor medications.

NAFLD: Non-alcoholic fatty liver disease; ALD: Auto-immune liver disease; ARLD: Alcohol-related liver disease; COVID-19: Coronavirus disease-2019; ERCP: Endoscopic retrograde cholangiopancreatography; GI: Gastrointestinal; PPE: Personal protective equipment; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; HBV: Hepatitis B virus; HCV: Hepatitis C virus; ACLF: Acute-on-chronic liver failure; ALD: Alcoholic liver disease; HCC: Hepatocellular carcinoma; AASLD: American Association for the Study of Liver Diseases; EASL: European Association for the Study of the Liver; ESCMID: European Society of Clinical Microbiology and Infectious Diseases; APSDE: Asian Pacific Society for Digestive Endoscopy; AGA: American Gastroenterological Association; ESGE: European Society of Gastrointestinal Endoscopy; ASGE: American Society for Gastrointestinal Endoscopy; ILTS: International Liver Transplantation Society; LTSI: Liver Transplant Society of India; ATS: American Thoracic Society; TTS: Transplantation Society; ILCA:

International Liver Cancer Association; ASCO: American Society of Clinical Oncology; ESMO: European Society for Medical Oncology.

Association for the Study of the Liver (EASL)[101], advocate the use of telemedicine to minimize interaction between patients and health workers by recommending prevention and management interventions that include sectioning of COVID-19 inpatients from other clinically healthy patients and optimization of the use of telemedicine. For COVID-19 adults with chronic disease (particularly if other risk factors are present), the EASL recommends considering early admission and with an upscaled biochemical profile, must be examined for concomitant viral hepatitis B or C infections[105]. In patients with strong clinical suspicion of deep venous thrombosis, or biliary blockage, diagnostic imaging must only be implemented in selected patients, and biopsies should be postponed, but certain cases should be admitted to detect autoimmune hepatitis[101,104,105]. Besides using EHR and telemedicine in some patients as an alternative to in-person care, various medical centers must change their modus operandi to meet the needs of social distancing[106].

Viral hepatitis-HCV and HBV: Medication for HCV and HBV should be administered following the general guidelines in patients without COVID-19[107]. If the patients are already taking medication such as anti-viral therapy for the treatment of chronic HCV/ HBV, telehealthcare, as well as clinical tests, should be conducted in addition to electronic follow-up prescriptions and if initiated, additional resources including a complete course of anti-viral medications with alternative therapies to prevent the uncertain effect of INF-a[53,54]. A case-by-case decision by the consultant should be taken for clients with COVID-19 and a high disease flare or a clinical concern of acute HBV hepatitis, and then patients should undergo antiviral therapy. The use of antiviral therapeutic interventions should be taken into account to avoid viral flares or reactivation in patients with severe, latent or healed HBV and COVID-19 treated with immunosuppressive agents[53].

Liver cirrhosis: The effect of SARS-CoV-2 infection on patients with cirrhosis and the negative impacts of late or amended treatment during the COVID-19 pandemic is especially important and every attempt must be made wherever possible to establish the highest quality treatment for cirrhosis patients in compliance with guidelines[53,108]. The option to postpone all regular screenings or monitoring procedures for patients with compensated cirrhosis should be available. As per the proposal of the Baveno VI consensus, it is recommended that low-risk patients should avoid intrusive variceal blood screening[106]. The risk of infection and associated comorbidities in cirrhosis patients is expanding, and is critical for patients who have decompensated cirrhosis, as a result of immune dysfunction related to cirrhosis. Particular measures should be carried out for those patients with cirrhosis admitted for reasons other than COVID-19 in a designated non-COVID-19 station, ideally side-rooms, in an attempt to mitigate the risk of SARS-CoV-2 nosocomial contamination[53]. Standardized rules and regulations to avoid and deter admission must also be strictly observed concerning GI hemorrhage, hepatic encephalopathy and prophylaxis of spontaneous bacterial peritonitis[53,108].

NAFLD or steatohepatitis: Metabolic complications such as hypertension, obesity and diabetes mellitus (DM) will increase the probability of severe COVID-19 in patients with NAFLD or steatohepatitis (NASH)[101,106]. The associated negative metabolic and hepatic effects due to social repression, including intensified sedentary habits and intake of convenience foods should be made known to patients[53,106]. The development of liver disease may be prevented by activities such as diet guidance, weight loss advice and managing DM, which may minimize severe disease following future infection with SARS-CoV-2. The treatment of arterial hypertension should follow current guidelines. There is currently no indication that angiotensin-converting enzyme blockers or angiotensin receptor antagonists increase either the risk of SARS-CoV-2 or the risk of serious complications or death from COVID-19. All patients with NAFLD and SARS-CoV-2 should be admitted to hospital promptly[53].

Specific recommendations for NAFLD: Patients with NAFLD are considered to be susceptible to COVID-19; therefore, specific recommendations should be followed. Patients with NAFLD should be mindful of the possible detrimental effects of social isolation on metabolic and hepatic function, as more unhealthy lifestyles and excessive intake of refined foods may exacerbate the condition. To prevent the progression of liver disease, good lifestyle choices should be made. Maintaining body weight and avoiding obesity can help to prevent future SARS-CoV-2 infection. Under current

guidelines, treatment of arterial hypertension should be continued as there is no research at present to suggest that angiotensin-converting enzyme inhibitors or angiotensin receptor blockers raise the risk of SARS-CoV-2 infection or the risk of experiencing serious complications or death from COVID-19. For all NAFLD patients who develop COVID-19 admission should be considered. Whenever possible, patients with NAFLD should be hospitalized in areas physically separated from other COVID-19 patients[53].

Alcohol-related liver disease: Alcohol intake can enhance the vulnerability of a person to SARS-CoV-2 infection. Social exclusion can contribute to new and elevated use of alcohol[109], a rise in alcohol consumption may be associated with further liver decompensation. Therefore, physicians and agencies should adopt preventive actions such as patient access to telephone alcohol liaison and abstinence. While therapy with corticosteroids has shown a positive benefit in hospitalized COVID-19 patients[110], there is still uncertainty as to whether patients already on elevated doses of corticosteroids are more likely to be seriously affected by COVID-19. These aspects should be addressed in patients with severe alcoholic hepatitis before the initiation of corticosteroids[53].

Autoimmune liver disease: Experts are currently suggesting that immunosuppression therapy should be avoided in COVID-19 patients with autoimmune liver disease. A decline in medication following lymphopenia/cytopenia or microbial super-infection in patients with severe COVID-19 should be considered only in exceptional circumstances following consultation with a physician[53,106]. Although corticosteroid treatment has had a positive effect in hospitalized COVID-19 patients, it is unknown whether patients treated with high doses of corticosteroids may be more vulnerable to SARS-CoV-2 infection and severe COVID-19. Therefore, we suggest the initial administration of budesonide to reduce systemic glucocorticoid exposure in patients with autoimmune hepatitis flare without cirrhosis[53,101]. Dexamethasone should be introduced or added only in COVID-19 patients who require hospital admission and respiratory assistance[110]. There is insufficient information on patients with IgG4-associated diseases, primary sclerosing cholangitis and biliary cholangitis to formulate specific guidelines. All patients should be vaccinated against *Streptococcus pneumoniae* and influenza[53,101].

Patients who have undergone liver transplantation: Patients with decompensated cirrhosis on the liver transplantation (LT) standby list are at greater risk of serious COVID-19 and death; therefore it is advisable that LT facilities should, as far as appropriate, be restored after the pandemic. Researchers urge the creation and enhancement of LT-donors and recipients' local and global risk pathways, which involve a combination of clinics. LT is a priority for those with a short-term prognosis, such as those with acute liver failure, elevated Model for End-stage Liver Disease score and hepatocellular carcinoma (HCC) at the top of the Milan criteria, in centers with constant resources[53,101,105]. It is currently proposed that all SARS-CoV-2 donors be tested using reverse transcription PCR before using SARS-CoV-2-infected donor livers[60]. It should be noted that patients with SARS infection waiting for LT are associated with a greater risk of COVID-19 and mortality following significant surgery; thus, the possible risk of nosocomial COVID should require consent for diagnostic and therapeutic procedures relating to transplantation[111]. Calcineurin-inhibitor drug levels and rapamycin inhibitor mechanistic targets should be closely monitored when used in combination with medications such as hydroxychloroquine, protease inhibitors or experimental COVID-19 drugs[53]. Case-by-case consideration should include diligent risk stratification of the donor (living) and recipient using a combination of the clinical background, chest X-rays and SARS-CoV-2 monitoring. A COVID-19-free transplant plan, including strict social isolation for patients on a housing list, wireless screening for signs and exposures before admission and peri-operative management in a designated clean intensive care facility and post-LT care unit should be developed in areas with high disease burden[53,101].

HCC: People living with cancer have worse COVID-19 consequences. This is likely to apply to HCC patients as they are usually older, more vulnerable and require several medications including cytotoxic chemotherapy[105,112]. Multi-factorial HCC boards may continue to operate remotely and offer clinical advice, including ongoing systemic treatment and LT assessment. When appropriate, full HCC monitoring shall be restored. Where resource constraints exist, priority should be given, by public HCC risk stratification ratings, to patients at higher risk, including those with elevated alpha-fetoprotein levels, advanced cirrhosis, chronic hepatitis B, NASH/diabetes,

etc[53,101].

Pharmacological management

COVID-19 is a fast-growing area for targeted management, with a wealth of new or repurposed drugs rapidly being in and out of favor. While no medications have been approved at present for SARS-CoV-2, in recent weeks many treatments have been tested and many are still are under investigation. It is encouraged to investigate possible hepatotoxic effects at "LiverTox" and drug interactions at "HEP drug interactions" before beginning any COVID-19 medications[106]. Unique factors in treatment trials for COVID-19 patients with chronic liver disease are briefly summarized here. Trials in COVID-19 patients include research on the following medications:

Remdesivir: Remdesivir is an adenosine nucleotide analogue demonstrated to minimize the length of symptoms following early use as a potential contender for COVID-19 therapy. It induced termination of the RNA chain and was first developed as an anti-Ebola agent[113]. In preclinical studies, remdesivir use in randomized studies demonstrated no major effect on liver function tests relative to a sugar pill, despite evidence of a reversible aminotransferase elevation. The elevation in aminotransferase was seen in patients on remdesivir after exclusion of certain liver conditions[53]. A clinical *in vitro* isolate of SARS-CoV-2 has recently been inhibited and in an *in-vivo* rhesus macaque model, the severity of the associated MERS-CoV infection was decreased by remdesivir[114].

Corticosteroids: The association between corticosteroids and COVID-19 seems inconclusive. Those with an existing serious disease appear to benefit from the addition of corticosteroids, while patients who have already taken corticosteroids may be more at risk of COVID-19 adverse effects[53]. Corticosteroids reduce pro-inflammatory cytokine production, and there is a risk of an increase in co-infection in persons with decompensated cirrhosis[106]. Their use has been related to intensive care unit admission, artificial ventilation or death in patients with inflammatory bowel disease. Similarly, the rheumatological hospitalization rating for maintenance glucocorticoids after SARS-CoV-2 infection has expanded[53]. Standard immunosuppression in patients with autoimmune hepatitis or LT, including steroids where necessary, is currently recommended. However, for patients with severe COVID-19 who require respiratory assistance, corticosteroids are a feasible treatment choice. Dexamethasone decreased mortality in perfusing patients by one-third and in patients who received additional oxygen by one-fifth in June 2020[110]. This agent is likely to be used more and more to treat serious COVID-19 even in patients with pre-existing chronic liver disease[53].

Anticoagulation: The risk of venous thromboembolism is greater in patients with advanced liver disease. The rate of venous thromboembolic disease in patients hospitalized due to COVID-19 is frighteningly high, with an observed 20% incidence on day 7, and 42% on day 21 despite thromboprophylaxis[115]. Therefore extensive analysis of the role of anticoagulation in COVID patients has demonstrated that the results in extreme COVID-19 are strengthened, although there are still coherent stepped care models and processing thresholds. While there are reservations regarding the use of anticoagulation in patients with liver cirrhosis and portal hypertension, there has been no systematic evaluation indicating excess blood disorders in cirrhosis- and portal vein thrombosis anticoagulated patients[53].

Tocilizumab: The main driver of the "cytokine storm," interleukin-6 (IL-6), appears to be significant in lungs and other organs due to severe COVID-19. Tocilizumab, a humanized monoclonal antibody targeting IL-7 has shown value in retrospective series of COVID-19 by reducing the need for and duration of organ support. As this agent is widely used in rheumatoid arthritis and other autoinflammatory diseases, its liver profile is well-established[53,101,106]. Mild serum aminotransferase elevations are common and are generally self-limited and asymptomatic. However, gradual jaundice requiring LT has been reported[53,106]. In rare cases, tocilizumab is associated with HBV reactivation[53].

Others: Several studies have indicated that other drugs have shown clinical effectiveness in patients with COVID-19. Clinical research in patients affected is underway to better determine their effectiveness. Chloroquine phosphate or hydroxychloroquine, ritonavir-boosted lopinavir, baricitinib *etc* are some of the

medications currently being assessed. Hydroxychloroquine impedes lysosomal acidification and autophagy, preventing *in-vitro* viral entry[106,116]. Baricitinib is a JAK 1/2-AAK1 inhibitor resulting in lymphopenia, HBV activation and is not recommended in those with liver impairment[106]. Drugs with natural origins have also been suggested to improve COVID-19-associated clinical manifestations including GI disturbances.

Limitations of existing therapy

At present, there is no evidence from clinical trials which shows the effectiveness of drugs in patients with either suspected or confirmed COVID-19, and there are no clinical trial results available that endorse prophylactic treatments. Therefore, repurposing old drugs is the only option to cope with the current pandemic until vaccines are developed. Chloroquine/hydroxychloroquine, lopinavir, ribavirin, remdesivir, favipiravir, corticosteroids, and tocilizumab are the only Food and Drug Administration approved medications for COVID-19. However, even after identifying old drugs for reutilization, there are several barriers to minimize the severity of COVID-19 such as dose adjustments, route of administration, mechanism of action, GI toxicity, and choice of delivery system to administer these old drugs. The limitations of using these agents are a tendency to cause acute heart and liver toxicity. This acute toxicity can overwhelm the undetermined advantage of a particular antiviral agent. Approximately 50% of patients treated with lopinavir experienced adverse reactions in a recent randomized controlled trial and 14% of patients discontinued treatment due to GI side-effects. Lopinavir may exacerbate hepatotoxicity and liver injury as it elevates alanine transaminase. Ribavirin causes severe dose-dependent hematological toxicity. High doses of ribavirin in SARS trials resulted in hemolytic anemia in more than 60% of patients. Tocilizumab has been linked to HBV reactivation and thus HBV serology should be part of the routine pre-treatment workup. The lack of clinical data suggesting a specific benefit of these agents do not explain their risks[53,117].

CONCLUSION

The recent COVID-19 pandemic has posed an unprecedented burden on human health. The lungs are the primary infection site for the causative agent of this COVID-19 pandemic, *i.e.*, SARS-CoV-2 and, therefore, initial investigations have mainly focused on its community spread and consequent pulmonary disorders. With the quantity of studies from various medical, bio-medical as well as allied fields, it is now established that the effects of SARS-CoV-2 are not limited to only the lungs. Systemic infection and pathological manifestations have been confirmed including those in the GI system. Considering the critical role of the GI system in physiological maintenance, it is essential to combat the SARS-CoV-2 infection-triggered anatomical damage. GI disturbances in COVID-19 also result in complications which are challenging in the clinical management of patients with co-morbidities including obesity, hyperglycemia, hypertensive disorders, liver diseases, cardiovascular disorders, *etc.* Although most of the symptoms in SARS-CoV-2 infected patients are similar to those known to arise in other respiratory viral infections, its novel nature and the degree of uncertainty regarding the outcome of therapeutic interventions make treatment challenging. Moreover, the steps taken to prevent the ongoing pandemic (social distancing, lockdown, stay-home strategies) also drive disturbances in physiological and mental well-being. This will contribute to GI anomalies due to altered daily routine, leisure activities, dietary habits, and hormonal imbalance (reduced vitamin D due to less sunlight exposure).

Due to its severity, rapid rate of spread and associated clinical complications, it is difficult to diagnose the GI ailments associated with COVID-19. Conventional methods and combinatorial strategies are suggested to provide an accurate diagnosis. The presence of SARS-COV-2 in various organs and fecal discharge, even in individuals negative for respiratory infections, indicates that GI organs also serve as a target and reservoir for the virus. As the GI system is involved in nutrient assimilation and physiological processes, viral infection results in diverse clinical manifestations in different systems including cardiovascular, neuropsychiatric, pulmonary, and hepatic, *etc.* Therefore, it is suggested that GI disturbances due to SARS-COV-2 infection must be considered as important as respiratory complications in COVID-19. This warrants the restructuring of medical service priorities to cover GI physiological disturbances in the treatment of COVID-19.

In the absence of any specific medication and prophylactic measures, the recent pandemic is expected to persist. However, the associated clinical manifestations of COVID-19 are not entirely known. Data on the clinical sequelae of COVID-19 in organs other than lungs are expected from ongoing investigations which are likely to increase. This requires the preparedness of health organizations to respond to the probable increase in GI disorders during and after the COVID-19 pandemic.

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ORIGINAL ARTICLE

Basic Study

Demethylation of miR-34a upregulates expression of membrane palmitoylated proteins and promotes the apoptosis of liver cancer cells

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Abstract

BACKGROUND

Liver cancer is a common cancer and the main cause of cancer-related deaths worldwide. Liver cancer is the sixth most common cancer in the world. Although miR-34a and palmitoyl membrane palmitoylated protein (MPP2) are reportedly involved in various cell processes, their precise roles in liver cancer are still unclear.

AIM

To investigate the expression of micro RNA 34a (miR-34a), methylation of the miR-34a promoter and the expression of MPP2 in liver cancer cells and their related mechanisms.

METHODS

Together, 78 cases of liver cancer tissues and 78 cases of adjacent tissues were collected. The methylation degree of miR-34a promoter in liver cancer/paracancerous tissue and liver cancer cells/normal liver cells, and the expression levels of miR-34a and MPP2 in the above samples were detected. Demethylation of liver cancer cells or transfection of liver cancer cells with miR-34a mimetic was performed. The MPP2 overexpression vector was used to transfect liver cancer

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cells, and the changes in proliferation, invasion, apoptosis, migration, and other biological functions of liver cancer cells after the above interventions were observed. Double luciferase reporter genes were used to detect the targeting relationship between miR-34a and MPP2.

RESULTS
Clinical samples showed that the expression levels of miR-34a and MPP2 in liver cancer tissues were lower than those in the normal tissues. The methylation degree of miR-34a promoter region in liver cancer cells was higher than that in normal liver cells. After miR-34a demethylation/mimetic transfection/MPP2 overexpression, the apoptosis of liver cancer cells was increased; the proliferation, invasion and migration capabilities were decreased; the expression levels of caspase 3, caspase 9, E-cadherin, and B-cell lymphoma 2 (Bcl-2)-associated X protein were increased; and the expression levels of Bcl-2, N-cadherin, and β-catenin were decreased. Double luciferase reporter genes confirmed that MPP2 is targeted by miR-34a. Rescue experiments showed that small interfering MPP2 could counteract the promoting effect of miR-34a demethylation on apoptosis and the inhibitory effect on cell proliferation, invasion, and migration.

CONCLUSION
miR-34a demethylation upregulates the expression level of MPP2 in liver cancer cells and promotes the apoptosis of liver cancer cells. miR-34a demethylation is a potential method for liver cancer treatment.

Key Words: Liver cancer; miR-34a; Membrane palmitoylated proteins; Methylation; Cell apoptosis; Caspase 3

Core Tip: This study confirmed the relationship of microRNA 34a (miR-34a) hypermethylation with membrane palmitoylated protein (MPP2) expression by investigating the changes of biological function and MPP2 expression level of liver cancer cells after miR-34a demethylation. miR-34a can inhibit the occurrence of liver cancer by upregulating MPP2, and its demethylation in liver cancer cells is a potential method of liver cancer treatment. In essence, miR-34a methylation is located upstream of its binding site with p53. Therefore, it is valuable to study the role of miR-34a methylation/demethylation on the expression level of upstream regulatory factors such as p53, and to explore its function in the future.

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INTRODUCTION

Liver cancer is the sixth most common cancer and second cause of cancer-related deaths worldwide[1]. In China, the population with a higher incidence of liver cancer is aged 50 or above[2]. Hepatitis B virus and hepatitis C virus are the main factors causing liver cancer[3]. The occurrence of liver cancer is a cumulative process. For example, liver cirrhosis mass develops from low to high, which can be regarded as the possible occurrence of liver cancer[4]. In the early stage of liver cancer, tumor resection, liver transplantation, local ablation and other treatment methods have relatively optimistic curative effects, but the recurrence rate of the above treatment in 5 years is relatively high[5]. MicroRNA (miRNA) influences the occurrence of tumors by mediating post-transcriptional regulation of gene expression[4], so targeted administration of miRNA has considerable prospects in tumor treatment.

miRNA is a key member of the non-coding RNA family[6], and can bind to the 3'-

untranslated region (UTR) of mRNA to regulate the expression of mRNA[7]. miR-34a plays an important role in cell apoptosis. Inhibition of miR-34a can promote cell proliferation[8]. Yamakuchi *et al*[9] found that miR-34a binds to the 3'-UTR segment of sirtuin 1 (SIRT1) in human colon cancer cells to inhibit SIRT1 expression and ultimately regulate cell apoptosis. Chang *et al*[10] believed that p53 trans-activates miR-34a, thus causing gene re-expression and promoting cell apoptosis. miR-34a is involved in the development of liver cancer. Cui *et al*[11] proved that the low expression of miR-34a is related to the large tumor of liver cancer patients and the high serum alpha-fetoprotein level. miR-34a was considered a predictor of early recurrence of liver cancer. In liver cancer, miR-34 was upregulated by enhancing emodin protein[12].

Membrane palmitoylated protein (MPP) contains many domains for cell-cell interaction[13]. MPP promotes the polarization of epithelial cells and the formation of tight junctions by forming complexes with human Discs Large 1[14]. At the same time, MPP also interconnects the cell membrane-cytoskeleton[15]. MPP may be a specific exosome accessory factor required for the maturation of ribosomal RNA[16], indicating that MPP may affect protein synthesis. Moreover, MPP2 can control cell growth and interfere with intracellular protein by enhancing E7 protein[17].

Comparing the expression levels of miR-34a in cancer tissues and paracancerous tissues, we found that miR-34a expression is low in liver cancer. Since abnormal DNA methylation at promoters is frequently involved in tumorigenesis, we explored the methylation of miR-34a promotor. Methprimer predicted that the promoter region of miR-34a has CpG binding sites. Methylation-specific polymerase chain reaction (PCR) found that miR-34a is hypermethylated in liver cancer. Based on this, we speculated that methylation may be the cause of low miR-34a expression in liver cancer, and we designed experiments for confirmation. miRDB and TargetScan databases revealed that MPP2 is downstream of miR-34a through exploration of the molecular mechanism of miR-34a methylation and MPP2 in liver cancer cells.

MATERIALS AND METHODS

Collection of tissue samples

Tissue samples were collected from 78 patients with liver cancer in Shandong Cancer Hospital affiliated to Shandong University (Shandong, China), including 42 males and 36 females. Inclusion criterion was as follows[4]: Liver cancer was confirmed using blood tests, imaging tests, and liver biopsy. Exclusion criteria were as follows[4]: mentally ill patients; patients with other tumors; patients with a treatment history of surgery, chemotherapy, radiotherapy or antibiotic treatment; and patients who did not cooperate with the treatment. The patients were fully informed of the study, and the study was approved by the Shandong Cancer Hospital affiliated to Shandong University Ethics Committee. Tissue samples and sections were stored in liquid nitrogen for later use.

Cell culture, transfection, and demethylation

Hepatocellular carcinoma cell lines (HepG2, Hep3B, PLC/PRG/5) and human normal hepatocytes (THLE-2) were purchased from ATCC (Manassas, VA, United States). The cells were cultured in a cell incubator in 37 ℃ with 5% CO_2. The culture medium system was as follows: Dulbecco's Modified Eagle Medium (DMEM; Hyclone, Logan, UT, United States) + 10% fetal bovine serum (FBS) solution (Gibco, Gaithersburg, MD, United States) + 1% penicillin/streptomycin solution (100X, Solarbio, Beijing, China). Subsequent experiments were carried out when the cell fusion reached 80%-90%.

Before transfection, the medium was replaced without FBS to eliminate interference. Transfected cells were inoculated into 6-well plates with 1 × 10^5 cells per well. MPP2 small interfering RNA (siRNA) (siMPP2), MPP2-short hairpin RNA (shRNA) (sh-MPP2), negative control (NC) shRNA, miR-34a mimics (pre-miR 34a), and NC mimics (NC pre) were all purchased from Shanghai Sangon Biotech (Shanghai, China). Lipofectamine 2000 Transfection Kit (Invitrogen, Carlsbad, CA, United States) was used for cell transfection. After transfection for 8 h, fresh culture medium was applied and cultured in a humidity incubator including 5% CO_2 with constant temperature for 48 h at 37 ℃.

Another group of cells with good growth status were treated with 5-Aza methylation inhibitor (10 mol/L). After 48 h, fresh culture medium was applied. The culture condition was 37 ℃ and 5% CO_2.

Extraction of total RNA and reverse transcription reaction

The tissue sample was cut, milled, and prepared into a cell suspension. Then 1 mL Trizol was added to the cell suspension, and a pipette was used for repeated inhalation, and blow-out of cell suspension was performed to facilitate full cell lysis and extract total RNA from the tissue samples. The concentration and purity of total RNA at 260-280 nm were detected using an ultraviolet spectrophotometer, and those with OD260/OD280 more than 1.8 were used in subsequent experiments. The FastQuant RT Super Mix (KR108) kit (Tiangen Biotech, Beijing, China) was selected for reverse transcription. Then 5 μL of 4 × FQ-RT Super Mix, 1 μg total RNA, and RNase-Free ddH_2O were used to replenish the system to 20 μL. Reverse transcription reaction was carried out for 15 min at 42 ℃ and enzyme inactivation was carried out for 3 min at 95 ℃.

Detection of methylation of miR-34a by methylation-specific PCR method

Methprimer was used to predict the CpG site of miR-34a promoter. DNA was treated with sulfite using EZ DNA Methylation-GoldTM Kit (Zymo Research, Irvine, CA, United States). Methylation-specific PCR (MSP) detection was carried out using the MSP Kit (Tiangen). The miR-34a MSP primer was designed and synthesized by Shanghai Sangon Biotech. Upstream primer of miR-34a-MSP (5' to 3'): TGTTAGTTTTTTCGGGGAGTTTTCGG; downstream primer of miR-34a-MSP (5' to 3'): ACGCCAACTCCTCCCCCGTCCCGAAC; upstream primer of miR-34a-UMSP (5' to 3'): TAGTTTTTTTGGGGAGTTTTTGGTTT; downstream primer of miR-34a-UMSP (5' to 3'): TAACACCAACTCCTCCCCCATCCCAA.

qPCR detection of miR-34a, MPP2 mRNA

miR-34a and MPP2 mRNA were quantified using FastFire qPCR PreMix SYBR Green Kit (Tiangen) and ABI PRISM 7000 (Applied Biosystems, Foster City, CA, United States) instruments. The primers were designed and synthesized by Shanghai Sangon Biotech. The upstream primer of miR-34a (5' to 3'): TCCTTCCTACTCG TACCACCAAA, and the downstream primer of miR-34a (5' to 3'): AGGTGAGGAGATGTCGTTGT; upstream primer of MPP2 mRNA (5' to 3'): AGATCT TCTTCTTCAAGGACCGGTT; downstream primer of MPP2 mRNA (5' to 3'): GGCTGGTCA GTGGCTTGGGGTA. The reaction system referred to the kit specification (50 μL). The reaction system was as follows: 1.5 μL upstream primer, 1.5 μL downstream primer, 25 μL of 2 × FastFire qPCR PreMix, 5 μL cDNA template, 5 μL of 50 × ROX Reference Dye, and RNase-Free ddH2O was added to make the total reaction system to 50 μL. The reaction process was as follows: pre-denaturation at 95 ℃ for 3 min, for 1 cycle; denaturation at 95 ℃ for 15 s, annealing at 60 ℃ for 15 s, for a total of 40 cycles. The results were analyzed by ABI PRISM 7000 instrument. The internal reference genes were U6 and GAPDH, which were standardized by $2^{-\Delta\Delta CT}$ method. The upstream primer of GAPDH (5' to 3'): GTCTCCTCTGACTTCAACAGCG, downstream (5' to 3'): GTCTCCTCTGACTTCAACAGCG. The upstream primer of U6 (5' to 3'): CGCTTCGGCAGCACATATAC, downstream (5' to 3'): AAAATA TGGAACGCTTCACGA.

Determination of protein expression by Western blotting

The protein was extracted and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrotransferred to nitrocellulose filter membrane, and placed at room temperature for 1 h (closed with 5% of skim milk -phosphate-buffered solution [PBS] solution). MPP2 (at 2 g/mL), caspase 3 (1:5000), caspase 9 (1:10000), E-cadherin (1:10000), B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) (1:10000), Bcl-2 (1:2000), N-cadherin (1:10000), β-catenin (1:10000) and β-actin (1:10000) primary antibodies were added and placed overnight at 4 ℃. The nitrocellulose membrane was washed with PBS solution for three times, then goat anti-rabbit secondary antibody (horseradish peroxidase [HRP] cross-linked) was added, and the mixture was placed for 1h at room temperature. Finally, the nitrocellulose membrane was washed with PBS solution and visualized using enhanced chemiluminescence method. The internal reference protein was β-actin, and the relative expression level of the protein to be detected was normalized to the intensity of β-actin band.

Caspase 9, caspase 3, E-cadherin, Bax, Bcl-2, N-cadherin, β-catenin, β-actin primary antibody and secondary antibody goat anti-rabbit (HRP cross-linked) were purchased from Shanghai Abcam Company (Shanghai, China).

Detection of cell survival by the MTT assay

Trypsin enzymolysis was added to cells, centrifugation was performed to remove

trypsin solution, fresh culture medium was added, and repeated inhalation and blow-out of cell suspension were carried out to prepare the cell suspension. Four 96-well plates were taken and cells were inoculated into the well (5×10^3 cells/100 μL per well), with three wells in each group. One well plate was taken out every 24 h, 5 mg/mL MTT solution was added with 10 μL/well, the cells were continued to culture for 1 h, then the culture medium was removed, and the OD value was measured at 570 nm with a microplate reader. Since the number of living cells is proportional to the absorbance, the absorbance value is applied as the cell activity. The experiment was repeated three times to visualize the cell activity-time curve.

Evaluation of cell migration and invasion by the Transwell method

Trypsin enzymolysis was carried out to prepare the cell suspension. Cells were inoculated into the migration upper chamber (containing 200 μL 10% FBS +1% DMEM) with 2×10^4 cells/well, and DMEM (containing 10% FBS with a total volume of 500 μL) was added into the lower chamber. After 24 h of cell culture, the upper chamber fluid was removed and the cells on the chamber wall were wiped off. A 4% of polymethanol was used to fix the Transwell opposite cells for 20 min. Crystal violet was used for staining for 15 min, and PBS was used to wash the Transwell chamber. Images of cell migration were collected under a 200-fold microscope. The cell number was calculated by randomly selecting three fields of view, and the average value was taken as the number of transmembrane cells. The experiment was repeated three times. Invasion was paved with 8% matrix glue on the above steps, and the number of cells per well was increased to 5×10^4.

Double luciferase reporter of gene determination

After amplification, MPP2 was cloned into pmirGLO vector to construct pmirGLO-MPP2-wt, and GeneArt TM Site-Directed Mutagenesis PLUS System (Thermo Fisher Scientific, Waltham, MA, United States) to construct pmirGLO-MPP2-mut vector, which was co-transfected with miR-34a, Ncmics, NC pre and pre-miR-34a into cells respectively. After 48 h of transfection, the luciferase intensity was detected using double luciferase reporter genes (Promega, Madison, WI, United States). The operation steps were carried out strictly in accordance with the instructions.

Statistics and analysis

SPSS 20.0 (AsiaAnalytics, Shanghai, China) was applied for index data analysis, and GraphPad Prism 6.0 was used for statistical analysis. Each experiment was repeated three times. The measurement data were expressed by mean ± SD. The comparison between groups was performed using one-way analysis of variance and *t*-test. Pearson correlation analysis was applied to discuss the correlation between miR-34a and MPP2. All data were tested using two-tailed test. The value of 95% was taken as confidence interval, and the difference was considered statistically significant at $^aP < 0.05$.

RESULTS

Low expression of miR-34a and MPP2 in hepatocellular carcinoma

In this study, 78 cases of liver cancer tissues and adjacent normal tissues were randomly selected as research objects. qPCR and Western blotting were used to determine the expression of miR-34a and MPP2 (Figure 1). Compared with adjacent normal tissues, miR-34a and MPP2 were lower in liver cancer tissues. Subsequently, the expression levels of miR-34a and MPP2 in three liver cancer cells (HepG2, Hep3B, PLC/PRG/5) and human normal liver cells (THLE-2) were detected. Compared with THLE-2, miR-34a and MPP2 were poorly expressed in liver cancer cells. The above results indicate that miR-34a and MPP2 are expressed at lower levels in liver cancer.

Low miR-34a expression in hepatocellular carcinoma due to methylation

To determine the cause of low expression of miR-34a in liver cancer, methylation of the miR-34a promoter was studied. First, Methprimer was used to predict the CpG site in the miR-34a promoter region. The results showed that there was a CpG site near the promoter 500 bp (Figure 2A). Then the MSP method was used to detect the methylation of miR-34a in tissue samples and cells. The results showed that methylated PCR bands appeared in liver cancer tissues and cells (Figure 2B). Based on this, we speculate that miR-34a expression is low in liver cancer due to methylation.

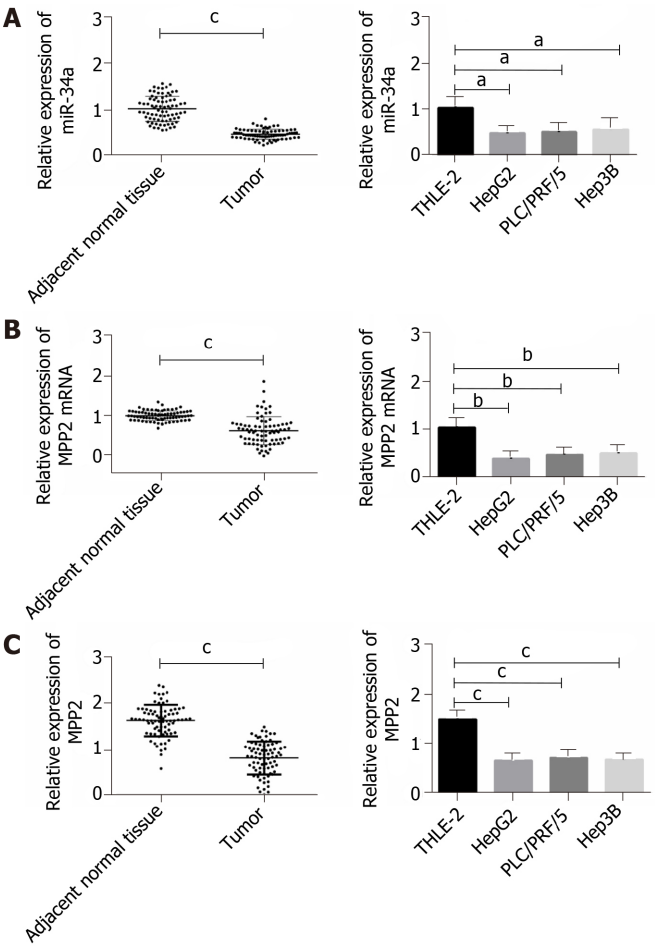


Figure 1 Low expression of microRNA 34a and membrane palmitoylated proteins in liver cancer. A: Low expression of microRNA 34a (miR-34a) in liver cancer tissues and cells; B: The mRNA expression of membrane palmitoylated protein (MPP2) was low in liver cancer tissues and cells; and C: The protein expression of MMP2 in liver cancer tissues and cells; a indicates $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$).

Upregulation of miR-34a promotes cell apoptosis and MPP2 expression, and inhibits cell proliferation, invasion, and migration

DNA methylation inhibitor 5-Aza was used to demethylate liver cancer cells, the MSP method was applied to detect the methylation of miR-34a, qPCR was applied to detect the expression level of miR-34a, and Western blotting was applied to detect the expression levels of MPP2, caspase 3, caspase 9, E-cadherin, Bax, Bcl-2, N- cadherin, and β-catenin. The MTT method was used to detect the changes in cell activity at different time points and concentrations. The Transwell method was used to detect the invasion and migration ability of cells. The specific results are shown in Figure 3 and Supplementary Figures 1 and 2. After 5-Aza intervention, the methylation level of miR-34a in cells was decreased and the expression level was increased. Compared with the NC demethylation group, the cell activity, cell migration, and proliferation were decreased; the expression of Bcl-2, N-cadherin and β-catenin was downregulated; and the expression levels of caspase 9, caspase 3, E-cadherin, and Bax were upregulated. Compared with the NC pre-group, the pre-miR-34a group also showed a similar change trend in miR-34a demethylation. After miR-34a demethylation or promotion of miR-34a expression level, MPP2 expression level was upregulated in liver cancer cells. The above results indicated that miR-34a expression level is upregulated after miR-34a demethylation, which promotes the expression and apoptosis of MPP2, caspase 9, caspase 3, E-cadherin, Bax, and Bcl-2 and inhibits cell proliferation, migration, and invasion.

Upregulation of MPP2 promotes cell apoptosis and inhibits cell proliferation, migration, and invasion

Since upregulation of miR-34a can change the expression level of MPP2, the effect of MMP2 overexpression on the biological function of liver cancer cells was studied. The MPP2 results showed that overexpression of MPP2 did not change the methylation

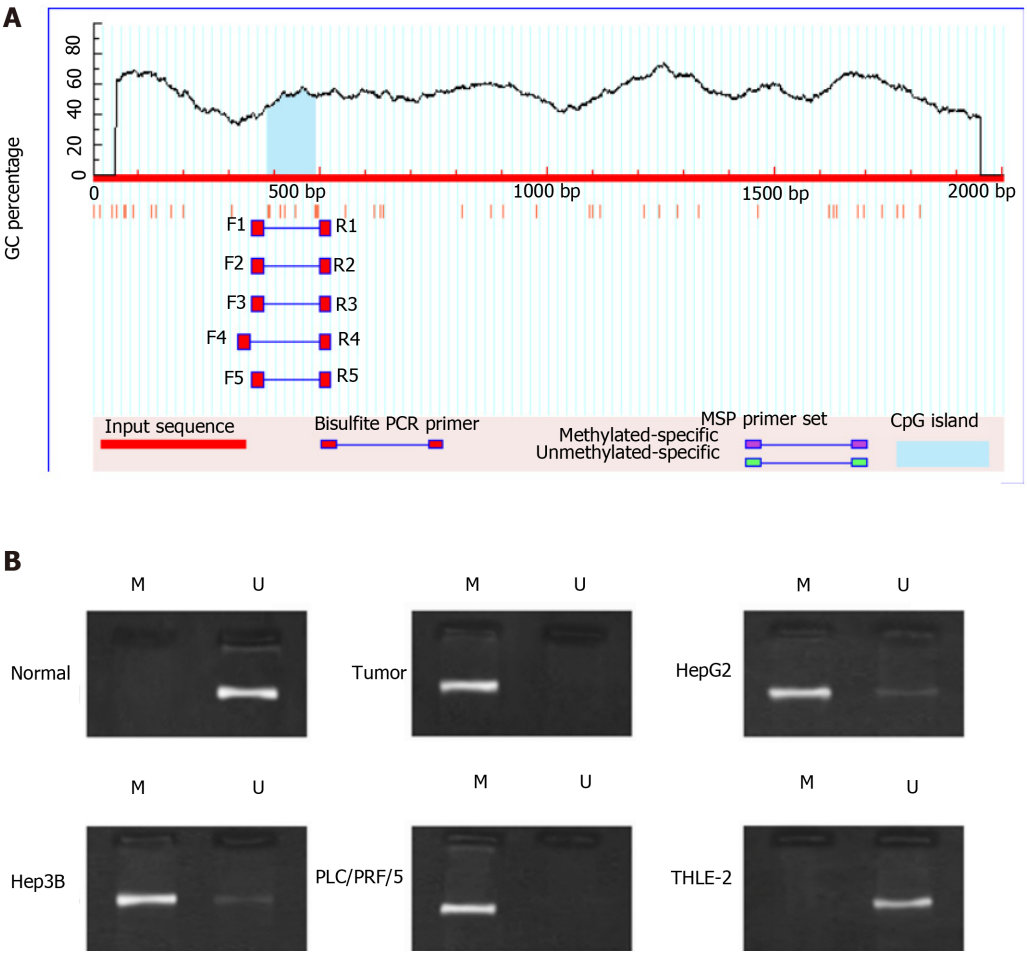


Figure 2 Highly methylated miR-34a in liver cancer. A: Methylation site prediction; B: Methylation of miR-34a in liver cancer. MSP: Methylation-specific polymerase chain reaction.

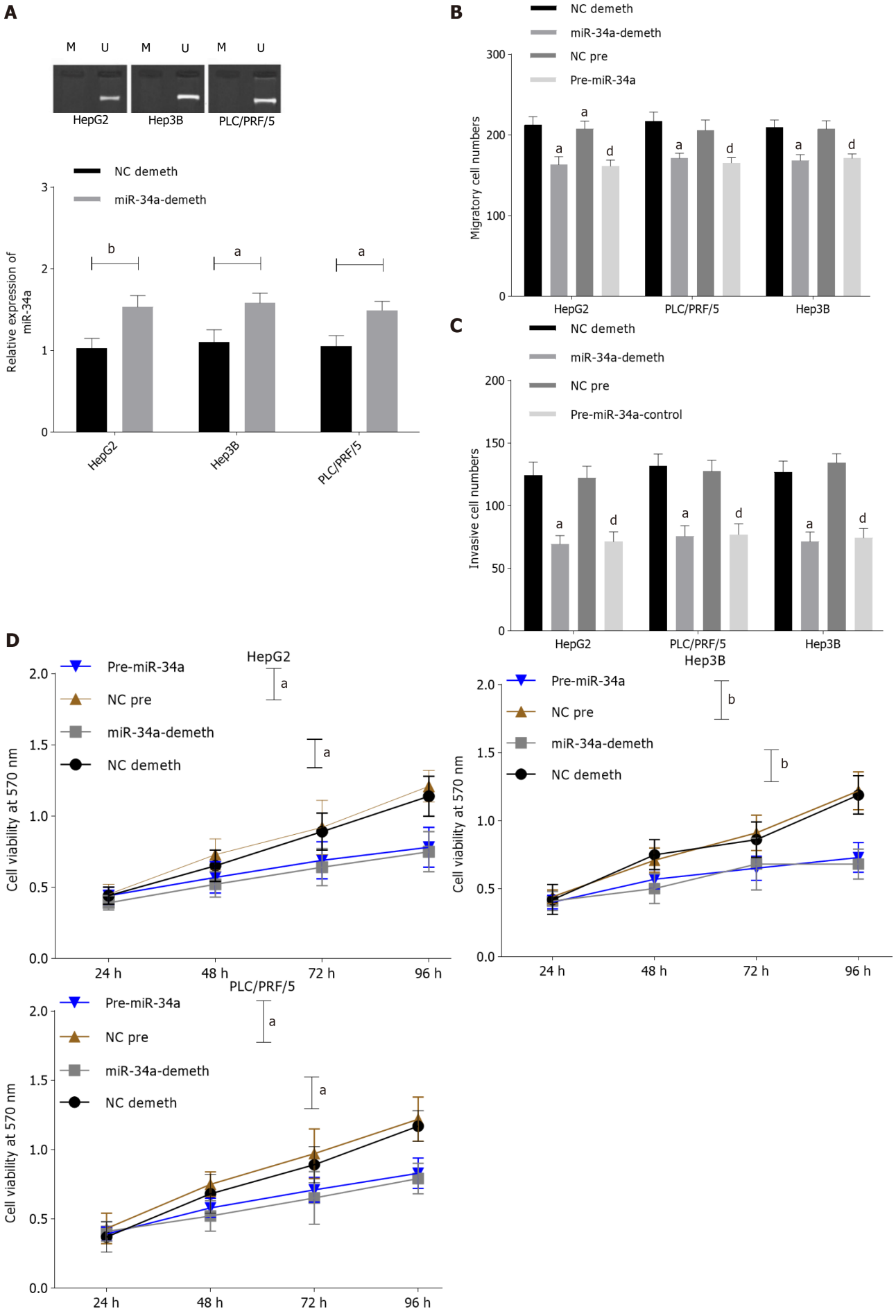
and expression level of miR-34a (Figure 4A). However, the Transwell experiment showed that compared with the NC-sh group, the cell migration number (Figure 4B) and invasion number (Figure 4C) of the sh-MPP2 group decreased significantly. The MTT assay showed that compared with the NC-sh group, the sh-MPP2 group showed a decrease in cell activity (Figure 4D). Western blot analysis showed that compared with the N- sh group, the expression levels of Bcl-2, N-cadherin, and β-catenin in the sh-MPP2 group were downregulated, whereas the expression levels of caspase 9, caspase 3, E-cadherin, Bax, and Bcl-2 were upregulated (Figure 4E). The above results indicate that upregulation of MPP2 promotes cell apoptosis and inhibits cell proliferation, migration, and invasion.

MPP2 is the target of miR-34a

Both the Targetscan and miRDB databases predicted that MPP2 was the target gene of miR-34a (Figure 5A). The upregulation or downregulation of MPP2 expression level was closely related to the upregulation or downregulation of miR-34a expression level (Figure 5B and C). Pearson analysis showed that there was a positive correlation between miR-34a and MPP2 (Figure 5D). To verif the targeted relationship between inflammatory miR-34a and MPP2, a double luciferase reporting experiment was carried out. The results showed that when miR-34a mimics and pmirGLO-MPP2-wt were co-transfected, fluorescence was decreased (Figure 5E). Therefore, miR-34a it appears can target and promote MPP2 expression.

Rescue experiment

The liver cancer cells were transfected with miR-34a demethylation and miR-34a demethylation + si MMP2 respectively, and corresponding MTT, Western blot analysis, and Transwell experiments were conducted (Figure 6, Supplementary Figures 3-5). Compared with the miR-34a demethylation group, cell migration and proliferation in the miR-34a demethylation + si MPP2 group were increased; cell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miR-34a-demeth
NC pre
Pre-miR-34a-control
Invasive cell numbers
HepG2 PLC/PRF/5 Hep3B
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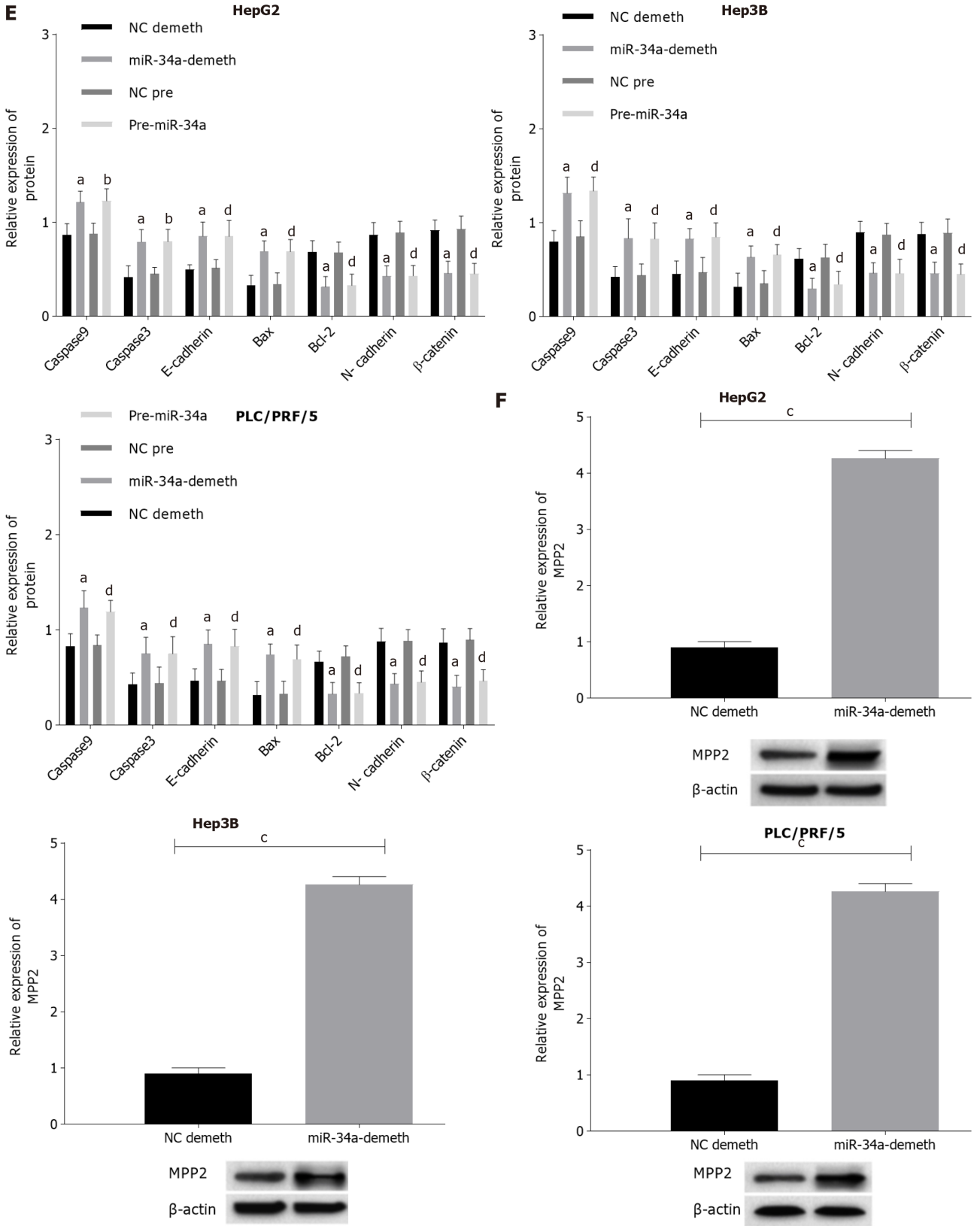


Figure 3 Upregulation of microRNA 34a promotes cell apoptosis and membrane palmitoylated protein expression, and inhibits cell proliferation, invasion, and migration. A: Image of microRNA 34a (miR-34a) demethylation results; B: Upregulation of miR-34a inhibits cell migration; a indicates that compared with negative control (NC) demethylation group, $^{a}P < 0.05$; $^{b}P < 0.01$, and d indicates compared with the NC pre group, $^{d}P < 0.05$; C: miR-34a upregulated the number of cell invasions; a indicates compared with the NC demethylation group, $^{a}P < 0.05$; d indicates compared with the NC pre group, $^{d}P < 0.05$; D: miR-34a upregulated and inhibited cell activity; a indicates $^{a}P < 0.05$, $^{b}P < 0.01$; E: Upregulation of miR-34a inhibited the downregulation of B-cell lymphoma 2 (Bcl-2), N- cadherin and β-catenin expression, while promoting caspase 9, caspase 3, E-cadherin and Bcl-2-associated X protein; a indicates compared with the NC demethylation group, $^{a}P < 0.05$; and d indicates compared with the NC pre group, $^{d}P < 0.05$; and F: Upregulation of miR-34a promoted the expression of membrane palmitoylated proteins, c indicates $^{c}P < 0.001$.

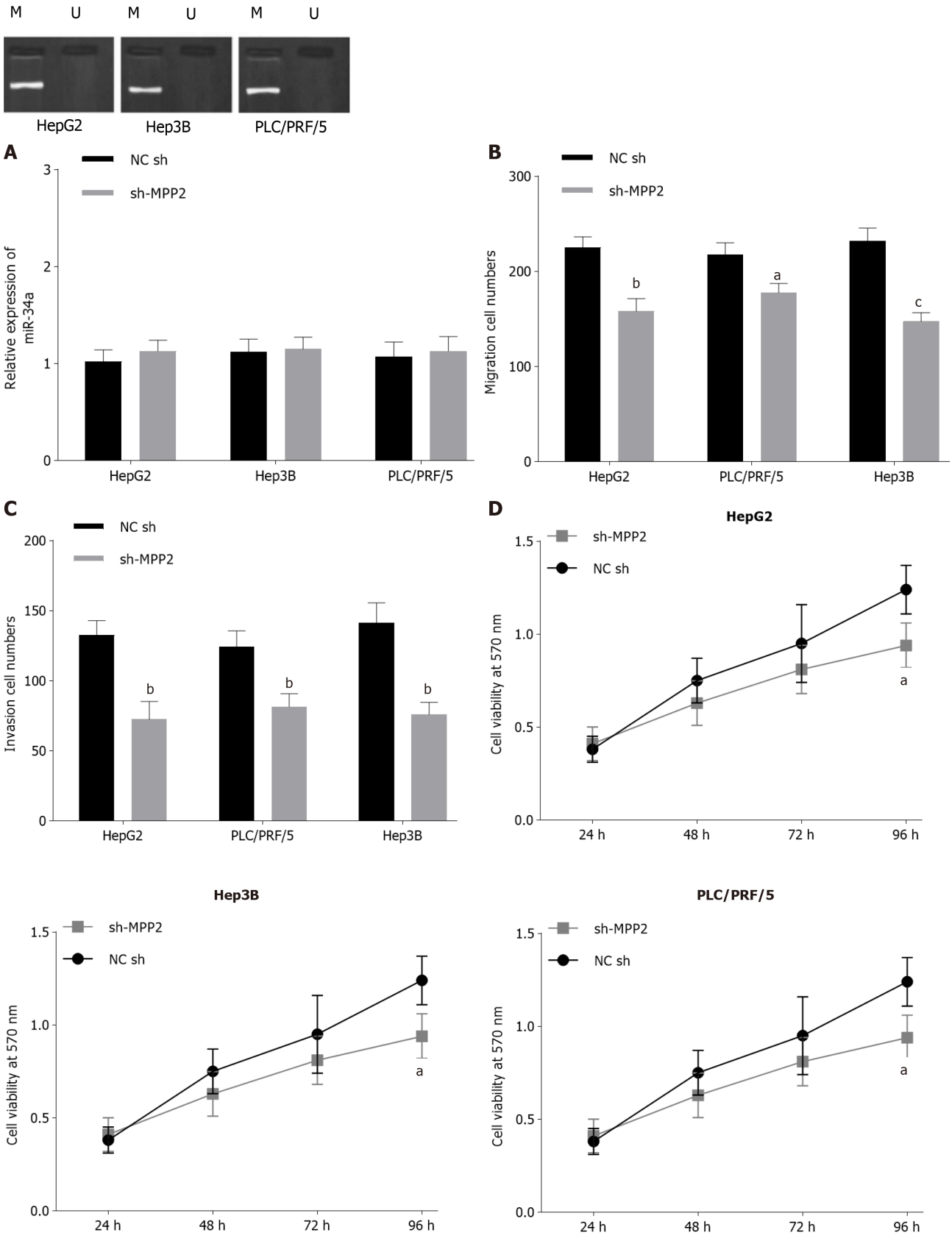
activity was increased; the expression levels of Bcl-2, N-cadherin, and β-catenin were upregulated; and the expression levels of caspase 9, caspase 3, E-cadherin, and Bax were downregulated. The miR-34a demethylation + si MPP2 group had no significant difference with the control group. The above results show that inhibition of MPP2 can counteract the effect of miR-34a demethylation on cell biological function.

DISCUSSION

miRNA plays an important role in the process of cell biology, and also affects cell processes such as cell proliferation, differentiation, apoptosis, and growth by regulating the expression level of many genes[18]. miRNA shows dynamic changes in different stages of cells. The high expression level of miRNA has specificity in different stages of cell development, thus playing a specific function in a certain period[19]. As the expression level of miRNA in normal tissues and tumor tissues is quite different[18] and can reflect the development and differentiation of tumor[20], how to regulate miRNA expression level has become a new research direction in cancer therapy. Some studies have claimed that miRNA is strictly regulated by DNA methylation[21]. Oshima *et al*[22] found that DNA methylation could downregulate miRNA encoded by 14q32 gene, thus inhibiting tumor development. Ning *et al*[23] showed that DNA (cytosine-5)-methyltransferase 1 and enhancer of zeste homolog 2 mediate DNA methylation and silence miR-200b, miR-200a and miR429, eventually promoting the development of gastric cancer and glioblastoma. Due to the reversibility of methylation and the amplification effect of miRNA, methylated miRNA can be used as a diagnostic index for cancer[24].

The miR-34a promoter has a CpG site near 400 bp to 500 bp (Figure 1B), so its expression level may be regulated by DNA methylation. The results showed that the expression level of miR-34a in liver cancer cells was lower than that in normal liver cells, whereas the methylation degree was higher than that in normal liver cells. After DNA demethylation, the expression level of miR-34a in liver cancer cells was increased; the apoptosis of cells was increased; and the migration, invasion, and proliferation ability were decreased. Many studies[25,26] have suggested that miR-34a can inhibit the proliferation and invasion of liver cancer cells and promote cell apoptosis by acting on different target proteins. The above results indicate that miR-34a is regulated by downregulation or silencing of CpG methylation[27], thus leading to the occurrence of liver cancer. The expression level of miR-34a in liver cancer cells was increased after demethylation, thus playing the role of tumor suppressor to prevent the subsequent development of liver cancer.

The essential action mechanism of miR-34a was to bind with downstream target protein mRNA, thus regulating transcription and translation of target gene and ultimately affecting cell biological function. To discuss the molecular mechanism of miR-34a on regulating cell biological functions, downstream studies of miR-34a were carried out. It was found that miR-34a had an MPP2 mRNA-binding site (Figure 5A), so miR-34a was considered a target on MPP2. The results showed that miR-34a could promote MPP2 expression in a targeted manner, thus causing increased apoptosis and decreased proliferation, migration and invasion of liver cancer cells. MPP2 belongs to the membrane-associated guanylate kinase p55 subgroup and plays an important role in cell-cell interaction[28]. The deletion and downregulation of MPP2 can lead to disorder of the SK2 channel and destroy Ca^{2+}/K^{+} diffusion, thus affecting cell signal transmission[29]. Baumgartner *et al*[30] showed that MPP2 plays a role as a tumor suppressor in tumorigenesis, which can inhibit the expression level of c-Src and affect cell proliferation, migration and invasion. Many studies[31-33] have confirmed that the miRNA regulation of genes is not limited to inhibitory effects, as miRNA can also promote gene expression by binding with mRNA. In this study, the effect of miR-34a on liver cancer may be that miR-34a binds to MPP2 mRNA, enhances mRNA transcription, and upregulates MPP2 expression. Upregulation of MPP2 will inhibit the expression level of downstream proteins such as c-Src, promote the recovery of pathways such as SK2, lead to changes in cytoskeleton of liver cancer cells, and eventually lead to weakening of malignant functions such as proliferation, migration and invasion. Cell adhesion plays an important role in cell migration and invasion, that is, cell adhesion inhibits epithelial-mesenchymal transition, which is the basis of cancer cell migration and invasion. MPP2 is a protein closely related to cell adhesion. In this study, the expression levels of intercellular adhesion molecules E-cadherin and N-cadherin were detected, which can be used to evaluate cell adhesion. Cancer cells are often accompanied by downregulation of E-cadherin and upregulation of N-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Migration cell numbers
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Invasion cell numbers
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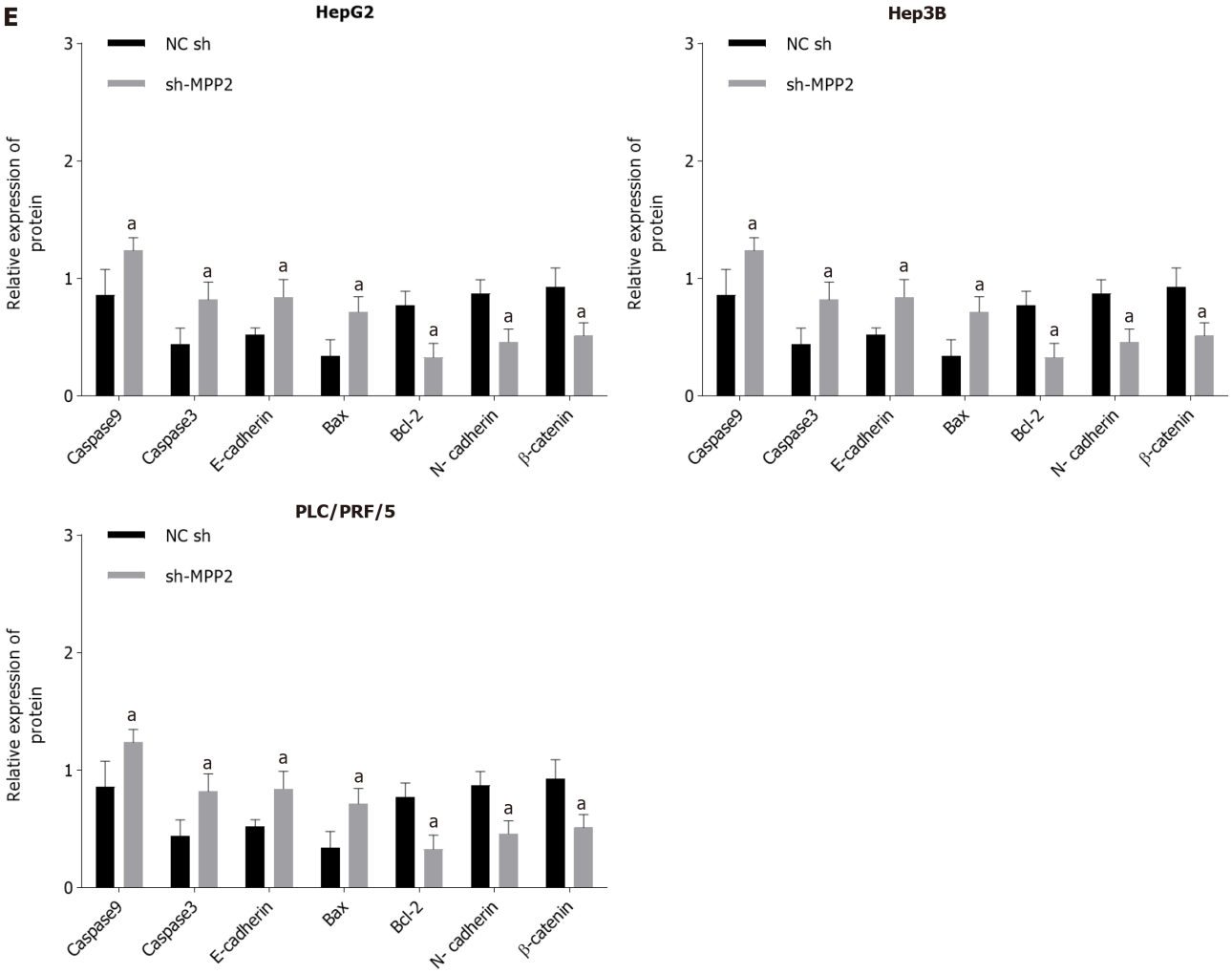


Figure 4 Membrane palmitoylated proteins promote cell apoptosis and inhibits cell proliferation, invasion, and migration. A: Overexpression of membrane palmitoylated proteins (MPP2) did not affect methylation and the expression level of microRNA 34a (miR-34a); B: MPP2 inhibited cell migration; compared with the negative control-short hairpin (NC-sh) group, a indicates $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$; C: MPP2 inhibited cell invasion, compared with the NC-sh group, b indicates $^{b}P < 0.01$; D: MPP2 inhibited cell activity compared with the NC-sh group, a indicates $^{a}P < 0.05$; and E: MPP2 inhibited the downregulation of B-cell lymphoma 2 (Bcl-2), N-cadherin and β-catenin expression, but promoted caspase 9, caspase 3, E-cadherin, Bcl-2-associated X protein compared with the NC-sh group, a indicates $^{a}P < 0.05$).

cadherin. In this paper, the demethylation of miR-34a or the upregulation of MPP2 could lead to the upregulation of E-cadherin and downregulation of N-cadherin. However, when miR-34a is demethylated and MPP2 is down-regulated, E-cadherin is downregulated and N-cadherin is upregulated. The above results indicate that demethylated miR-34a may promote cell adhesion through MPP2, and finally inhibit cell migration and invasion. Therefore, the relationship between miR-34a methylation and MPP2 expression can be used as a research point for liver cancer treatment. For the development of miR-34a, demethylation drugs are expected to become a potential treatment for hepatocellular carcinoma. Due to time and cost reasons, this paper failed to launch a suitable in vivo tumor experiment to further study the regulation of demethylated miR-34a/MPP2 axis in solid hepatocellular carcinoma. In addition, miRDB and TargetScan databases predicted that MPP2 might be the downstream target protein of miR-34a, and the double luciferase reporter gene verified that there was a targeting relationship between the two. However, other downstream target genes of miR-34a were not mentioned in this study. This is also the limitation of this paper. Other downstream targets of miR-34a will be discussed in future studies. According to Figure 1, miR-34a and MPP2 are statistically different between normal adjacent tissues and tumor tissues, indicating that miR-34a and MPP2 expression seem to be related to the distance between them and tumor. The lack of exploration on this point is the deficiency of this paper. Therefore, this "expression-distance" relationship will be discussed in depth in future research.

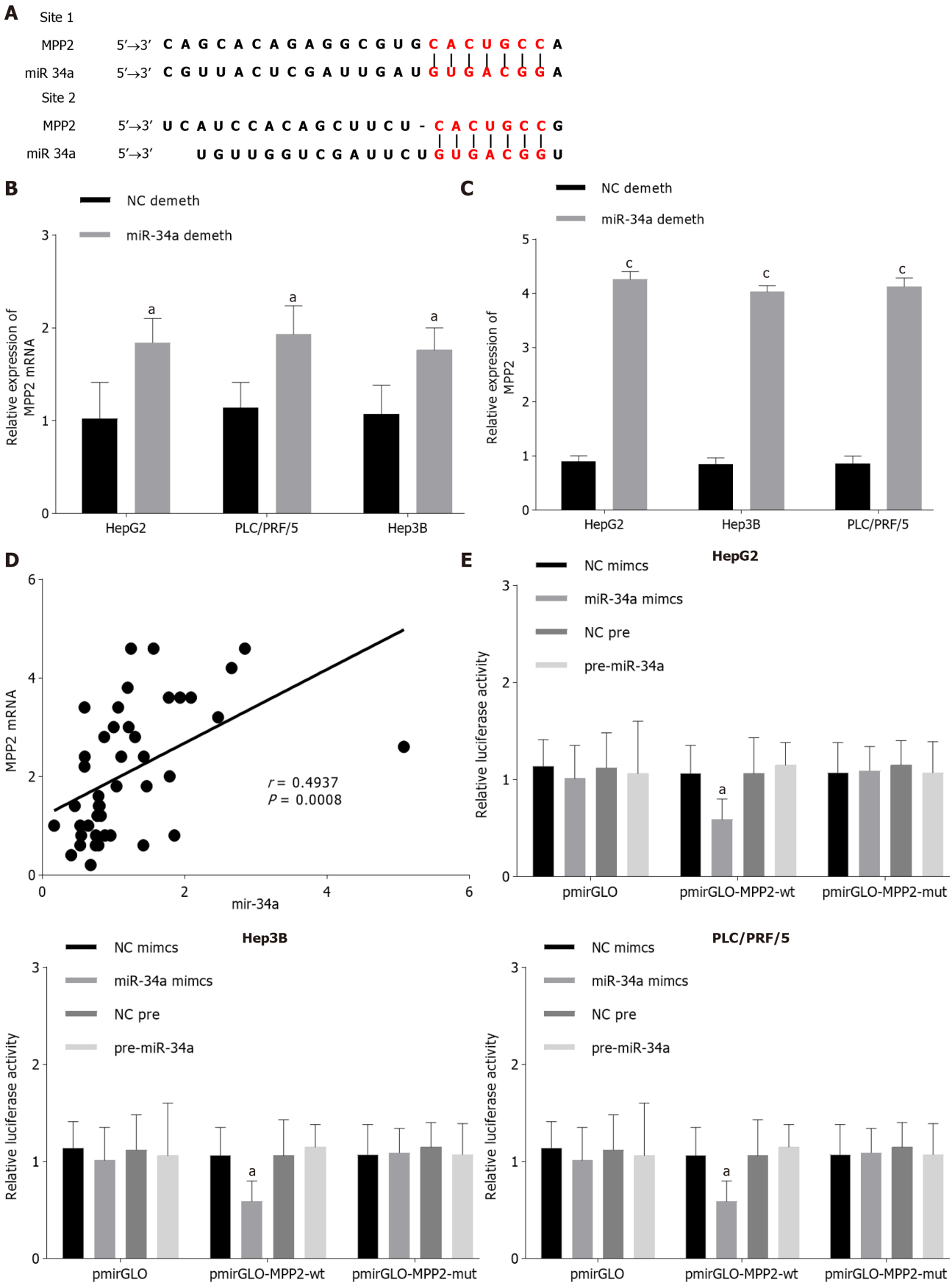
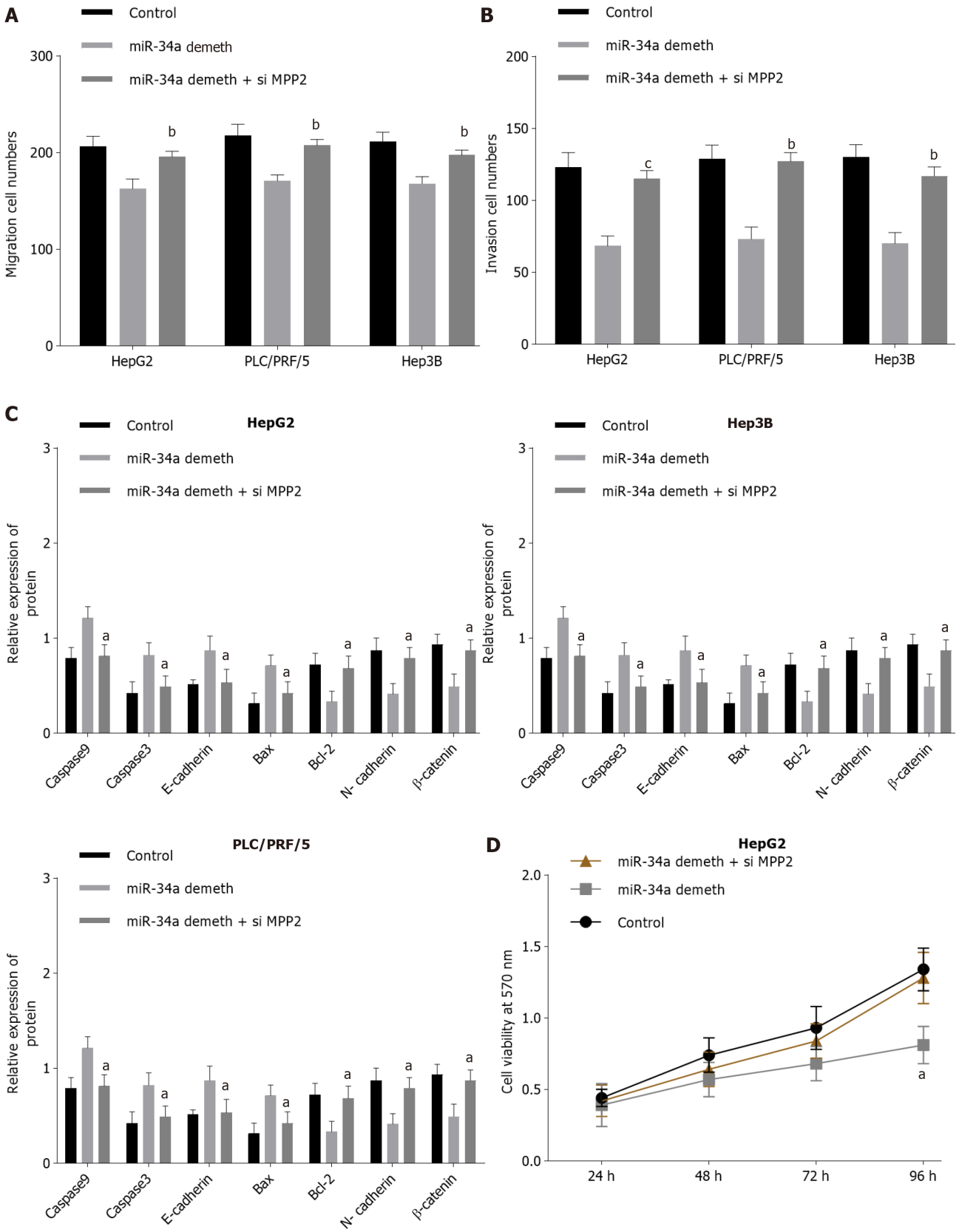


Figure 5 Membrane palmitoylated protein is the target of miR-34a. A: MicroRNA 34a (miR-34a) and membrane palmitoylated protein (MPP2) mRNA had binding sites; B and C: Effect of miR-34a expression change on MPP2 compared with the negative control (NC) demethylation group, a indicates $^{a}P < 0.05$; D: miR-34a had a positive correlation with MPP2 mRNA, $^{a}P < 0.05$, $^{c}P < 0.001$; and E: Double luciferase reporter gene verified the targeting relationship of miR-34a/MPP2; compared with NC mimics, a indicates $^{a}P < 0.05$.


A
Control
miR-34a demeth
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Migration cell numbers
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miR-34a demeth + si MPP2
Invasion cell numbers
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Relative expression of protein
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Caspase9
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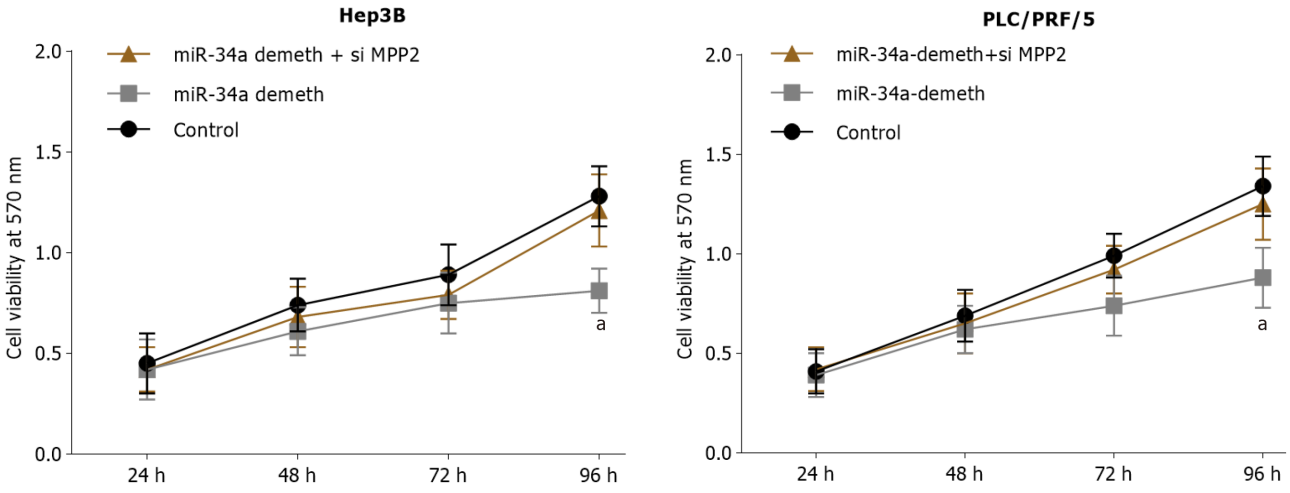

Figure 6 Rescue experiment. A: Small interfering membrane palmitoylated proteins (si-MPP2) can counteract the effect of microRNA 34a (miR-34a) demethylation on cell migration; B: si-MPP2 counteracts the effect of miR-34a demethylation on cell invasion, $^{b}P < 0.01$, $^{c}P < 0.001$; C: si-MPP2 counteracts the effect of miR-34a demethylation on cell activity; and D: si-MPP2 counteracts the effect of miR-34a demethylation on related proteins, a indicates $^{a}P < 0.05$).

CONCLUSION

This study identified the relationship of miR-34a hypermethylation with MPP2 expression by studying the changes of biological function and MPP2 expression of liver cancer cells after miR-34a demethylation. It was shown that miR-34a can inhibit the occurrence of liver cancer by upregulating MPP2, and its demethylation in liver cancer cells is a potential method for liver cancer treatment. In essence, the miR-34a methylation site is located on the upstream of its binding site with p53[27]. Therefore, it is valuable to study the role of miR-34a methylation/demethylation on the expression of upstream regulatory factors such as p53, and to explore its function in the future.

ARTICLE HIGHLIGHTS

Research background

Liver cancer is the sixth most common cancer in the world. Although miR-34a and palmitoyl membrane protein (MPP2) are reportedly involved in various cell processes, their precise roles in liver cancer are unknown.

Research motivation

miR-34a expression is low in liver cancer. The promoter region of miR-34a has CpG-binding sites and miR-34a is hypermethylated in liver cancer. MPP2 was downstream of miR-34a *via* miRDB and TargetScan databases prediction.

Research objectives

This study investigated the expression of miR-34a, the methylation of miR-34a promoter, and the expression of MPP2 in liver cancer cells and their related mechanisms.

Research methods

The methylation degree of miR-34a promoter and the expression levels of miR-34a and MPP2 in the above samples were detected. miR-34a was demethylated by 5′-Za. miR-34a or MPP2 was upregulated by miR-34a mimics or MPP2 shRNA. The changes in proliferation, invasion, apoptosis, migration and other biological functions of liver cancer cells were observed. Double luciferase reporter genes were used to detect the targeting relationship between miR-34a and MPP2.

Research results

miR-34a and MPP2 were downregulated in liver cancer and miR-34a was highly methylated in liver cancer. After miR-34a demethylation/mimetic transfection/MPP2 overexpression, liver cancer cells apoptosis was increased, but the proliferation,

invasion and migration capabilities were decreased. MPP2 was targeted by miR-34a.

Research conclusions

miR-34a demethylation upregulates the expression of MPP2 in liver cancer cells and promotes the apoptosis of liver cancer cells.

Research perspectives

The demethylation of miR-34a is a potential method for liver cancer treatment.

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ORIGINAL ARTICLE

Basic Study

Circular RNA circVAPA promotes chemotherapy drug resistance in gastric cancer progression by regulating miR-125b-5p/STAT3 axis

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Abstract

BACKGROUND

Gastric cancer (GC) is a prevalent malignancy, leading to a high incidence of cancer-associated death. Cisplatin (DDP)-based chemotherapy is the principal therapy for clinical GC treatment, but DDP resistance is a severe clinical challenge and the mechanism remains poorly understood. Circular RNAs (circRNAs) have been identified to play crucial roles in modulating the chemoresistance of gastric cancer cells.

AIM

To explore the effect of circVAPA on chemotherapy resistance during GC progression.

METHODS

The effect of circVAPA on GC progression and chemotherapy resistance was analyzed by MTT assay, colony formation assay, Transwell assay, wound healing assay, and flow cytometry analysis in GC cells and DDP resistant GC cell lines,

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and tumorigenicity analysis in nude mice *in vivo*. The mechanism was investigated by luciferase reporter assay, quantitative real-time PCR, and Western blot analysis.

RESULTS

CircVAPA expression was up-regulated in clinical GC tissues compared with normal samples. CircVAPA depletion inhibited proliferation, migration, and invasion and increased apoptosis of GC cells. The expression of circVAPA, STAT3, and STAT3 downstream genes was elevated in DDP resistant SGC7901/DDP cell lines. CircVAPA knockdown attenuated the DDP resistance of GC cells. Mechanically, circVAPA was able to sponge miR-125b-5p, and miR-125b-5p could target STAT3 in the GC cells. MiR-125b-5p inhibitor reversed circVAPA depletion-enhanced inhibitory effect of DDP on GC cells, and STAT3 knockdown blocked circVAPA overexpression-induced proliferation of DDP-treated SGC7901/DDP cells. The depletion of STAT3 and miR-125b-5p inhibitor reversed circVAPA depletion-induced GC cell apoptosis. Functionally, circVAPA contributed to the tumor growth of SGC7901/DDP cells *in vivo*.

CONCLUSION

CircVAPA promotes chemotherapy resistance and malignant progression in GC by miR-125b-5p/STAT3 signaling. Our findings present novel insights into the mechanism by which circVAPA regulates chemotherapy resistance of GC cells. CircVAPA and miR-125b-5p may be considered as the potential targets for GC therapy.

Key Words: Gastric cancer; Progression; Chemoresistance; CircVAPA; miR-125b-5p; STAT3

Core Tip: We concluded that circVAPA contributed to chemotherapy resistance and malignant progression in gastric cancer (GC) by miR-125b-5p/STAT3 signaling. Our findings present novel insights into the mechanism that circVAPA regulates chemotherapy resistance of GC cells. CircVAPA and miR-125b-5p may be considered as potential targets for GC therapy.

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INTRODUCTION

Gastric cancer (GC) is a prevalent malignancy and the second cause of tumor-associated mortality in the world[1]. Chemotherapy is a favored strategy toward advanced-stage GC cases, in which oxaliplatin, cisplatin (DDP), and 5-FU are usually admitted as first-line therapies[2-4]. Although the improved chemotherapy effectiveness, the GC patients' survival is still unsatisfactory, and chemotherapy always attains a low response rate[5,6]. Whether intrinsic or acquired, chemoresistance is a significant challenge for GC treatment efficiency in the clinic[7]. The mechanism for GC chemotherapy resistance development is not well-understood, in which micro-environmental and genetic contributors are able to result in chemoresistance[8]. Hence, understanding the molecular mechanism of chemotherapy resistance in GC is urgently needed.

Circular RNAs (circRNAs), as an emerging class of non-coding RNAs, present various functions in the regulation of gene expression at the post-transcriptional and transcriptional levels[9]. It has been identified that circRNAs are able to serve as the competitive endogenous sponge for microRNAs (miRNAs) and thereby inhibit

miRNAs, providing a new manner for modulating miRNAs and indicating a promising mechanism of circRNA function[10,11]. Meanwhile, previous studies have reported that circVAPA is elevated in tumors and contributes to cancer progression, such as liver cancer and breast cancer[12,13]. MiRNAs are short non-coding RNAs with a length of about 22nt and can target various genes by pairing with the mRNAs at the post-transcriptional level[14,15]. MiR-125b-5p is a well-investigated tumor suppressor in multiple cancer models, including bladder cancer, laryngeal squamous cell carcinoma, and colon cancer[16-18]. Furthermore, it has been well-identified that circRNAs and miRNAs are involved in the chemotherapy resistance in GC[8,19-21]. However, the role of circVAPA and miR-125b-5p in modulating chemotherapy resistance of GC cells is still unreported.

It has been recognized that STAT3 serves as a critical regulator in tumorigenesis[22]. As an important transcriptional factor, STAT3 is able to mediate multiple gene expression in modulating cell survival response to different stimulations[23]. Furthermore, STAT3 remarkably promotes chemoresistance as a crucial indicator in GC cells by various mechanisms, such as increasing the expression of vacuolar ATPase pump and oncogenes as well as the apoptotic factors[24]. Nevertheless, the impact of circVAPA and miR-125b-5p on STAT3 in the regulation of GC cells chemotherapy resistance is still unclear.

In this study, we were interested in the function of circVAPA in the progression of chemotherapy resistance of GC cells. We found an unreported role of circVAPA in enhancing chemotherapy resistance and promoting the malignant progression of GC by modulating miR-125b-5p/STAT3 signaling.

MATERIALS AND METHODS

GC clinical samples

The clinical GC tissue samples (n = 50) and adjacent normal tissues (n = 50) were obtained from the First Affiliated hospital of China Medical University. The diagnosis of cases was performed by two clinicians based on histopathological analysis. The samples were collected and immediately stored at -80 °C followed by further analysis. The samples were written-approved by the patients. This study conformed to the experimental guidelines of the World Medical Association and the Ethics Committee of the First Affiliated Hospital of China Medical University.

Cell culture and treatment

The SGC7901, HGC27, and SGC7901/DDP cell lines were maintained in the laboratory. The cells were incubated in an incubator at 5% CO_2 and 37 °C in the Dulbecco's modified Eagle's medium (DMEM) medium (Hyclone, United States) with fetal bovine serum (10%, Hyclone, United States), streptomycin (0.1 mg/mL, Hyclone, United States), and penicillin (100 units/mL, Hyclone, United States). DDP was obtained from Sigma (United States). The circVAPA shRNA, pcDNA3.1-circVAPA, pcDNA3.1-STAT3, STAT3 siRNA, miR-125b-5p mimic, and miR-125b-5p inhibitor were purchased from GenePharma (China). The transfection in the cells was performed with Liposome 2000 (Invitrogen, United States).

MTT assay

The cell viability was assessed by MTT assays at various time points in 6-well dishes. Briefly, the MTT solution (Solarbio, China) was added into the cells and cultured at the condition of 5% CO_2 and 37 °C for 4 h. After that, dimethyl sulfoxide (DMSO; 100 μL, 10 min; Sigma, United States) was used to terminate the reaction. The cell viability was analyzed at the absorbance of 490 nm by applying a microplate reader (Thermo, United States).

Colony formation assay

About 1 × 10^3 cells were plated in 6-well dishes and cultured in DMEM at the condition of 5% CO_2 and 37 °C. After 2 wk, cells were washed with phosphate buffered saline (PBS) buffer, made in methanol about 30 min, and dyed with 1% crystal violet dye solution, after which the number of colonies was calculated.

Transwell assay

Transwell assays were performed to analyze cell invasion and migration using a Transwell plate (Corning, United States) according to the manufacturer's instructions.

Briefly, the upper chamber was plated with around 1 × 10^5 cells. The cells were then fixed with 4% paraformaldehyde and dyed with crystal violet. The invaded and migrated cells were recorded and calculated.

Wound healing assay

Cells were plated in a 24-well plate at 3 × 10^5/well and cultured overnight to reach full confluence as a monolayer. A 20 μL pipette tip was applied to slowly cut a straight line across the well. Then the well was washed with PBS three times and changed with the serum-free medium and continued to culture. The wound healing percentage was calculated.

Analysis of cell apoptosis

Cell apoptosis was measured by applying the Annexin-V-FITC apoptosis kit (BD, United States) based on flow cytometry analysis using a FACSCalibur flow cytometer, followed by the quantification analysis using FlowJo software.

Luciferase reporter gene assay

SGC7901 and HGC27 cells were plated in 24-well dishes followed by treatment with miR-125b-5p mimic. After 48 h of the treatment, the luciferase activities were analyzed by utilizing the luciferase reporter assays (Promega, United States). The relative luciferase activities were analyzed by the Renilla luciferase activities.

Quantitative real-time PCR

RNA isolation was performed by applying TRIzol reagent (Solarbio, China) and the first-strand cDNA was synthesized (Solarbio, China). Quantitative real-time PCR was carried out by applying SYBR-Green (Takara, China). The primer sequences are as follows: circVAPA forward: 5′-TGGATTCCAAATTGAGATGCGTATT-3′, reverse: 5′-CACTTTTCTATCCGATGGATTTCGC-3′; miR-125b-5p forward: 5′-TCCCTGAGACCCTAACTTGTGA-3′, reverse: 5′- AGTCTCAGGGTCCGAGGTATTC-3′; STAT3 forward: 5′-CTGGCCTTTGGTGTTGAAAT-3′, reverse: 5′-AAGGCACCC ACAGAAACAAC-3′; GAPDH forward: 5′-AAGAAGGTGGTGAAGCAGGC-3′, reverse: 5′-TCCACCACCCAGTTGCTGTA-3′.

Western blot analysis

Total proteins were extracted from the cells using radioimmunoprecipitation assay buffer (CST, United States) and quantified using the BCA Protein Quantification Kit (Abbkine, United States). The proteins at the same concentration were subjected to sodium dodecyl sulfate- polyacrylam-ide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, United States), followed by incubation with 5% milk and with the primary antibodies at 4 °C overnight. The corresponding second antibodies (BOSTER, China) were used for incubating the membranes for 1 h at room temperature, followed by visualization using a chemiluminescence detection kit (Beyyotime, China). The primary antibodies applied in this study comprised STAT3 (Abcam, United States), p-STAT3 (Abcam, United States), Survivin (Abcam, United States), Mcl-1 (Abcam, United States), Bcl-xl (Abcam, United States), and β-actin (Abcam, United States).

Analysis of tumorigenicity in nude mice

The tumor growth of GC cells *in vivo* was tested in nude Balb/c mice (4-week-old, male, n = 5). And about 1 × 10^7 SGC7901/DDP cells were subcutaneously injected in the mice. Five days after injection, we measured tumor growth every 5 d. We sacrificed the mice 30 d after injection, and tumors were scaled. Tumor volume (V) was observed by estimating the length (L) and width (W) with calipers and calculated as (L × W^2) × 0.5. Animal care and method procedure were authorized by the Animal Ethics Committee of the First Affiliated Hospital of China Medical University.

Statistical analysis

Data are presented as the mean ± SD, and statistical analyses were performed with GraphPad Prism 7. The unpaired Student's t-test was applied for comparing the difference between two groups, and one-way ANOVA was applied for comparing among multiple groups. $^aP < 0.05$ were considered statistically significant.

RESULTS

CircVAPA enhances proliferation and represses apoptosis of GC cells

To identify the clinical association of circVAPA with GC, we analyzed the expression of circVAPA in the clinical GC samples. Our data showed that circVAPA expression was elevated in the GC tissue samples (n = 50) relative to that in the adjacent normal tissues (n = 50), implying the potential correlation of circVAPA with GC (Figure 1A). The cell viability was reduced by the depletion of circVAPA in the SGC7901 and HGC27 cell lines (Figure 1B and C). Similarly, colony formation assays revealed that circVAPA shRNA treatment remarkably decreased the colony numbers of SGC7901 and HGC27 cells (Figure 1D and E). Furthermore, apoptosis of SGC7901 and HGC27 cells was induced by circVAPA knockdown (Figure 1F and G). These findings indicated that circVAPA is able to increase proliferation and attenuate apoptosis of GC cells

CircVAPA promotes invasion and migration of GC cells

Next, the function of circVAPA in modulating migration and invasion of GC cells was assessed. Transwell assays demonstrated that migration and invasion of SGC7901 and HGC27 cells were alleviated by circVAPA shRNA (Figure 2A and B). Besides, wound healing assays showed that circVAPA depletion remarkably increased wound proportion in SGC7901 and HGC27 cell lines (Figure 2C and D). These data indicated that circVAPA contributes to migration and invasion of GC cells.

Elevation of circVAPA enhances chemotherapy drug resistance of GC cells

Next, we were interested in the impact of circVAPA on the chemoresistance of GC cells. To this end, we first explored the expression of circVAPA in SGC7901 and DDP resistant SGC7901/DDP cell lines. Significantly, the expression of circVAPA was up-regulated in SGC7901/DDP cells compared with that in SGC7901 cells (Figure 3A). Meanwhile, STAT3 phosphorylation, STAT3 expression, and STAT3 downstream gene (including Survivin, Mcl-1, and Bcl-xl) expression were elevated in the SGC7901/DDP cells relative to those in SGC7901 cells (Figure 3A and B). Importantly, depletion of circVAPA remarkably reduced the IC_{50} value of DDP for the inhibition of SGC7901/DDP cells (Figure 3C). In addition, DDP was able to induce apoptosis of SGC7901/DDP cells, and circVAPA depletion notably reinforced this effect in the system (Figure 3D). Together, these findings suggested that circVAPA contributes to chemotherapy drug resistance of GC cells.

CircVAPA is able to sponge miR-125b-5p in gastric cancer cells

Next, we explored the mechanism of circVAPA-mediated GC progression and chemotherapy resistance. The bioinformatic analysis revealed the potential interaction of circVAPA and miR-125b-5p (Figure 4A). The SGC7901 and HGC27 cells were treated with miR-125b-5p mimic and the enhancement of miR-125b-5p was confirmed in the cells (Figure 4B). Treatment with miR-125b-5p mimic was able to decrease luciferase activities of wild type circVAPA, but not circVAPA mutant in the cells (Figure 4C and D). Moreover, the expression of miR-125b-5p was up-regulated by circVAPA shRNA in the SGC7901 and HGC27 cells. These findings indicated that circVAPA spongs miR-125b-5p in the GC cells (Figure 4E and F).

MiR-125b-5p targets STAT3 in GC cells

Furthermore, we identified the binding site of miR-125b-5p within the 3′ untranslated region of the *STAT3* gene through bioinformatic analysis (Figure 5A). Treatment with miR-125b-5p mimic repressed luciferase activities of STAT3, but not STAT3 mutant in the SGC7901 and HGC27 cells (Figure 5B). Consistently, the mRNA and protein levels of STAT3 were inhibited by the miR-125b-5p mimic in the cells (Figure 5C and D). Moreover, circVAPA knockdown down-regulated STAT3 in the SGC7901 and HGC27 cells, while miR-125b-5p inhibitor was able to reverse this effect in the cells (Figure 5E).

CircVAPA promotes chemotherapy drug resistance of GC cells by modulating miR-125b-5p/STAT3 axis

We then investigated whether circVAPA mediates chemotherapy drug resistance of GC cells by modulating the miR-125b-5p/STAT3 axis. Significantly, depletion of circVAPA enhanced the inhibitory effect of DDP on SGC7901 and HGC27 cell viability, while miR-125b-5p inhibitor rescued this phenotype in the cells (Figure 6A and B). In addition, overexpression of circVAPA promoted the proliferation of DDP-

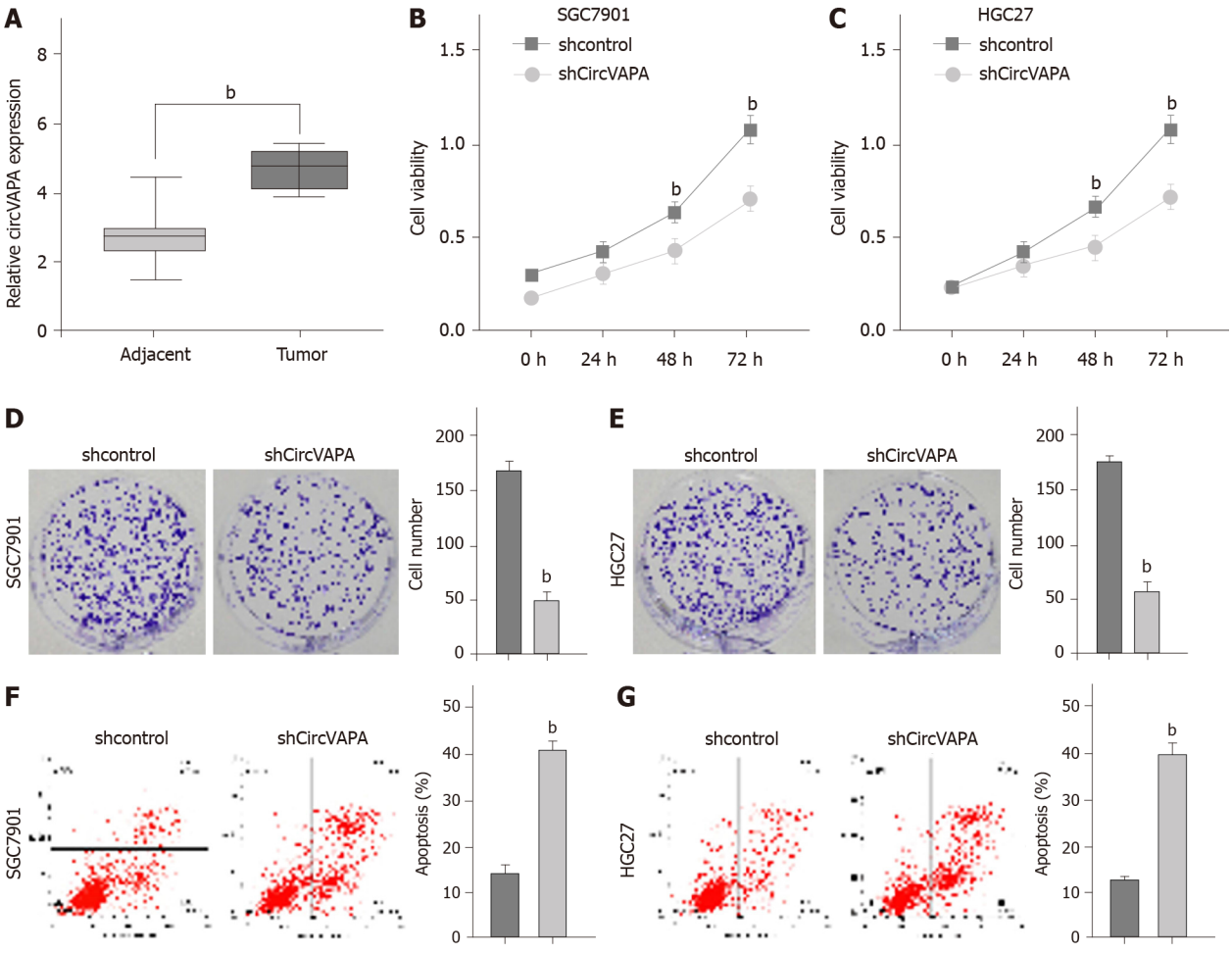


Figure 1 CircVAPA enhances proliferation and represses apoptosis of gastric cancer cells. A: The expression of circVAPA was measured by qPCR in the gastric cancer tissues (n = 50) and adjacent normal tissues (n = 50); B-G: SGC7901 and HGC27 cells were treated with the circVAPA shRNA or control shRNA. Cell viability was tested by MTT assay (B and C), cell proliferation was measured by colony formation assay (D and E), and cell apoptosis was analyzed by flow cytometry (F and G). Data are presented as the mean ± SD. $^{b}P < 0.01$.

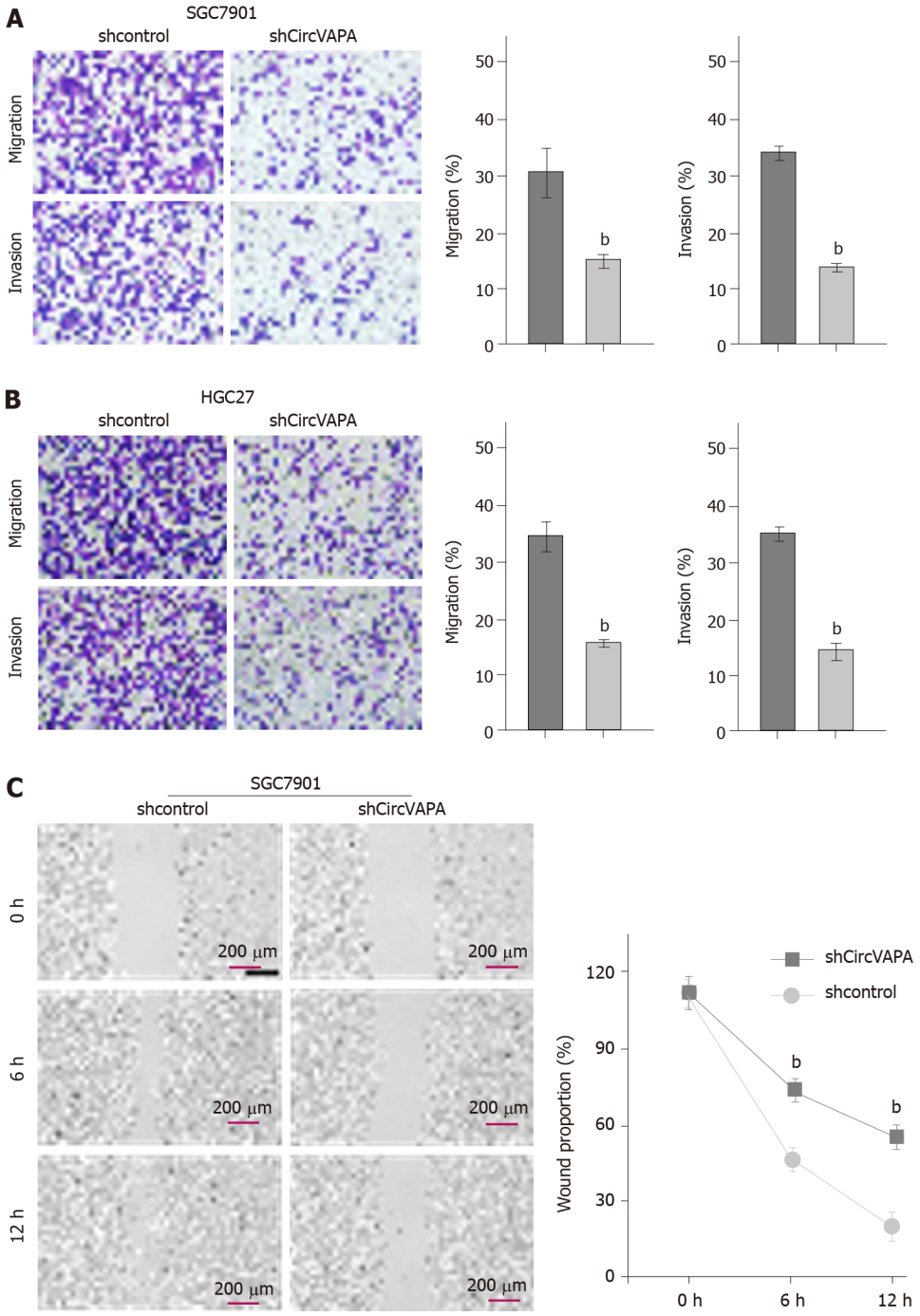
treated SGC7901/DDP cells, while STAT3 siRNA could reduce this effect in the system (Figure 6C). Meanwhile, circVAPA knockdown reinforced DDP-induced apoptosis of SGC7901 and HGC27 cells, whereas miR-125b-5p inhibitor or STAT3 overexpression could reverse this effect (Figure 6D). These results suggested that circVAPA promotes chemotherapy resistance of GC cells by modulating the miR-125b-5p/STAT3 axis (Figure 6E).

CircVAPA promotes the tumor growth of chemoresistant gastric cancer cell in vivo

Next, the circVAPA function in GC cell growth was analyzed by tumorigenicity analysis *in vivo*. The tumor growth of GC cells was inhibited by circVAPA knockdown, as presented by the decreased tumor size, tumor volume, and tumor weight in the nude mice (Figure 7A-C). Meanwhile, we validated that miR-125b-5p expression was up-regulated while STAT3 expression was down-regulated by circVAPA depletion in the tumor tissues (Figure 7D and E). Together, these data indicated that circVAPA contributes to the tumor growth of chemoresistant GC cells *in vivo*.

DISCUSSION

The chemotherapy resistance in GC patients is a severe clinical challenge, and the molecular mechanism is still poorly understood[7]. CircRNAs have been identified to participate in the modulation of GC progression[25]. Nevertheless, the specific functions of circVAPA in the modulation of GC development remain obscure. In the present study, we uncovered that circVAPA contributed to chemotherapy resistance and promoted the malignant progression in GC by modulating miR-125b-5p/STAT3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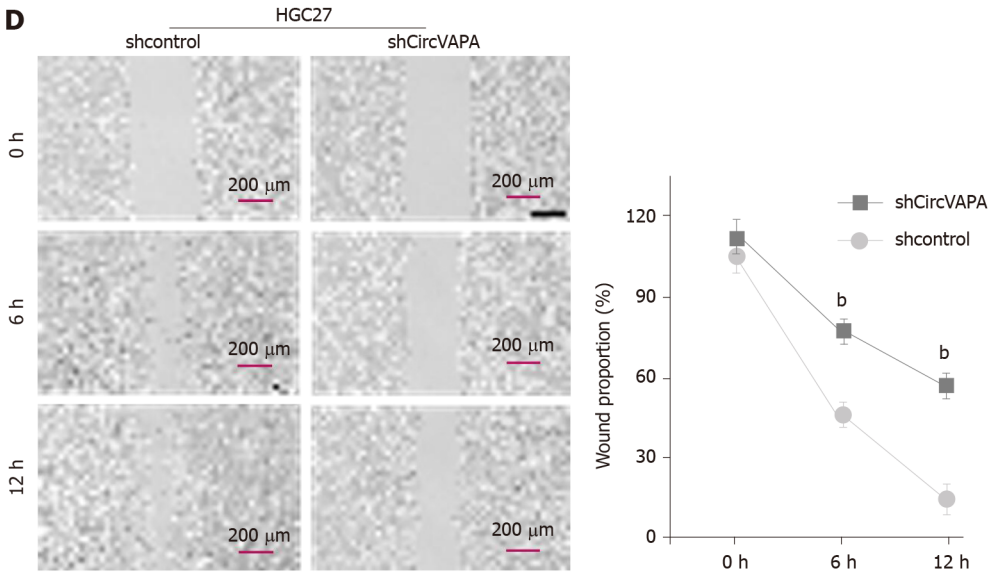

Figure 2 CircVAPA promotes invasion and migration of gastric cancer cells. A-D: SGC7901 and HGC27 cells were treated with the circVAPA shRNA or control shRNA. Cell migration and invasion were tested by transwell assay (A and B), and migration and invasion were analyzed by wound healing assay (C and D). The wound healing proportion is shown. Data are presented as the mean ± SD. $^{b}P < 0.01$.

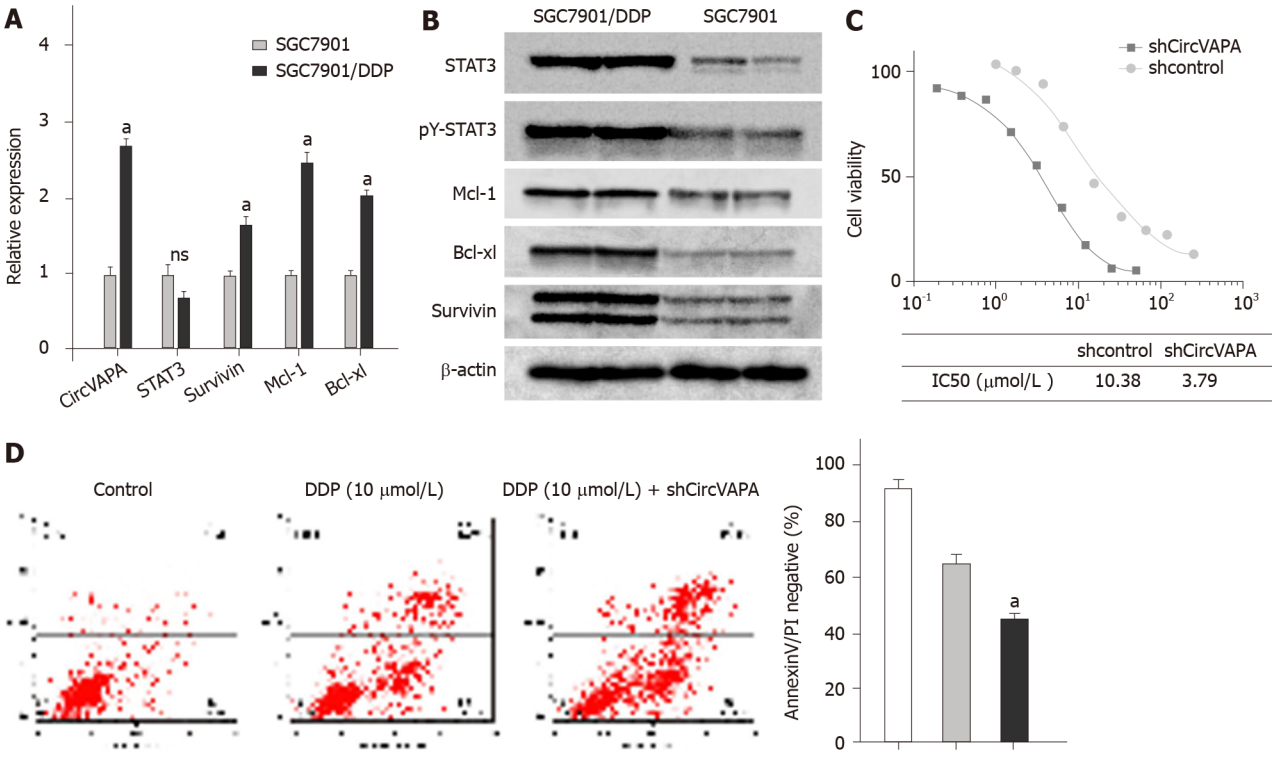

Figure 3 Elevation of CircVAPA enhances chemotherapy drug resistance of gastric cancer cells. A: The expression of circVAPA, STAT3, Survivin, Mcl-1, and Bcl-xl was measured by qPCR in the SGC7901 and SGC7901/cisplatin (DDP) cells; B: STAT3 phosphorylation and levels of STAT3, Survivin, Mcl-1, and Bcl-xl were tested by Western blot analysis; C: SGC7901/DDP cells were initially treated with DDP at indicated doses and then with control shRNA or circVAPA shRNA. Cell viability was analyzed by MTT assay; D: SGC7901/DDP cells were treated with DDP or co-treated with DDP and circVAPA shRNA. Cell apoptosis was analyzed by flow cytometry. Data are presented as the mean ± SD. $^{a}P < 0.05$.

signaling.

As a critical cancer regulator, several circRNAs have been identified in the modulation of chemotherapy resistance during GC progression. It has been reported that circ-PVT1 promotes paclitaxel resistance by regulating miR-124-3p/ZEB1 in GC cells[26]. Circ0026359 increases the DDP resistance of GC cells by modulating the miR-1200/POLD4 axis[27]. CircAKT3 promotes DDP resistance by up-regulating PIK3R1

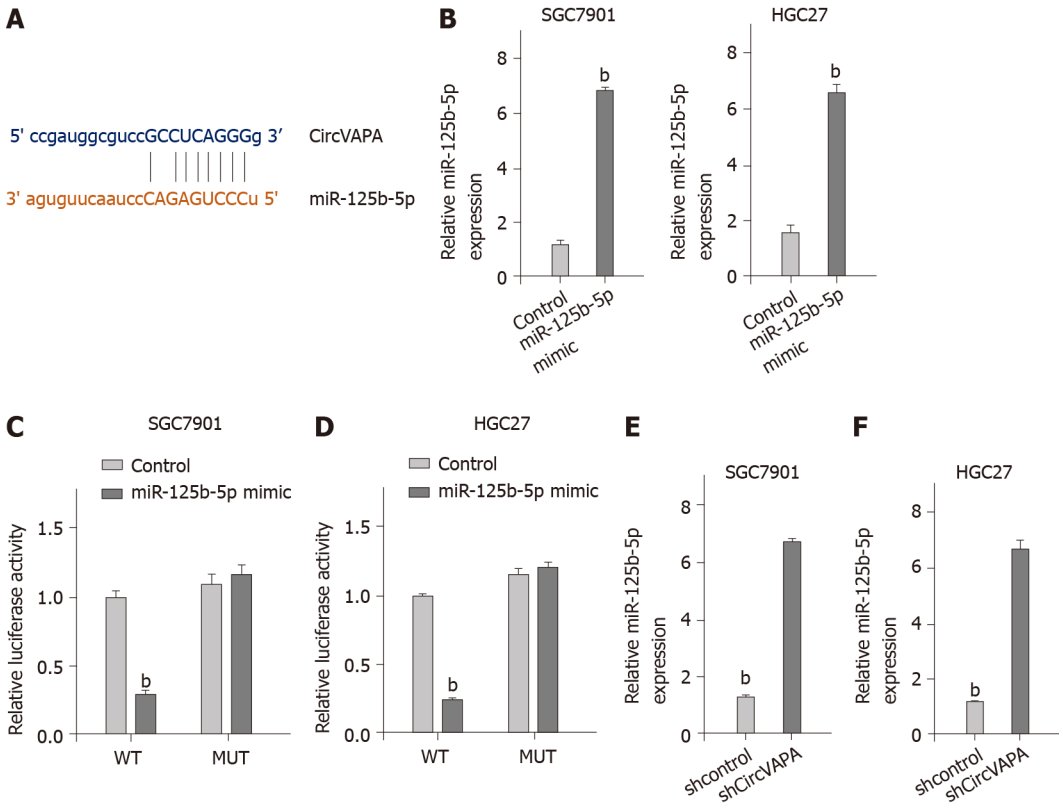

Figure 4 CircVAPA is able to sponge miR-125b-5p in gastric cancer cells. A: The interaction of CircVAPA and miR-125b-5p was analyzed by bioinformatic analysis based on ENCORI (http://starbase.sysu.edu.cn/index.php); B-D: SGC7901 and HGC27 cells were treated with control mimic or miR-125b-5p mimic. The expression levels of miR-125b-5p were tested by qPCR in the cells (B), and luciferase activities of wild type CircVAPA and CircVAPA with the miR-125b-5p-binding site mutant were examined by luciferase reporter gene assay (C and D); E and F: SGC7901 and HGC27 cells were treated with the circVAPA shRNA or control shRNA. The expression of miR-125b-5p was analyzed by qPCR assay. Data are presented as the mean ± SD. $^{b}P < 0.01$. MUT: Mutant; WT: Wild type.

through targeting miR-198 in GC cells[28]. CircFN1 contributes to DDP resistance of GC cells by targeting miR-182-5p[29]. CircMCTP2 attenuates DDP resistance by miR-99a-5p/MTMR3 signaling in GC cells[30]. Moreover, circVAPA presents oncogenic properties in cancers, including liver cancer and colorectal cancer[13,31]. In this study, circVAPA expression was elevated in the clinical GC samples. CircVAPA depletion inhibited proliferation, migration, and invasion, and increased apoptosis of GC cells. CircVAPA knockdown reduced tumor growth of GC cells *in vivo*. CircVAPA contributed to chemotherapy drug resistance of GC cells. These findings present a novel function of circVAPA in modulating chemotherapy drug resistance and malignant progression of GC cells, indicating valuable evidence of circRNA function in GC development.

MiRNAs are the essential contributor for regulating chemotherapy drug resistance of GC cells. It has been reported that miR-625 inhibits chemotherapy resistance by regulating ALDH1A1 in GC cells[32]. MiR-21-5p regulates doxorubicin resistance of GC cells *via* modulating TIMP3 and PTEN expression[33]. MiR-187 affects DDP resistance by targeting transforming growth factor-β signaling in GC cells[34]. Moreover, constitutive activation of STAT3 contributes to chemotherapy resistance of GC cells[35]. STAT3 signaling is involved in fibroblasts-mediated chemoresistance of GC cells[36]. Suppression of STAT3 inhibits drug resistance of GC cells to chemotherapeutic drugs[37]. In the mechanistic study, we found that circVAPA sponged miR-125b-5p and miR-125b-5p targeted STAT3 in the GC cells. MiR-125b-5p inhibitor rescued circVAPA depletion-enhanced inhibitory effect of DDP on GC cells, and STAT3 knockdown blocked circVAPA overexpression-induced proliferation of DDP-treated SGC7901/DDP cells. Depletion of STAT3 and miR-125b-5p inhibitor reversed circVAPA depletion-induced GC cell apoptosis. These data demonstrate an unreported regulation axis associated circVAPA with STAT3 and miR-125b-5p, revealing a new mechanism involving the circVAPA/miR-125b-5p/STAT3 axis in GC development.

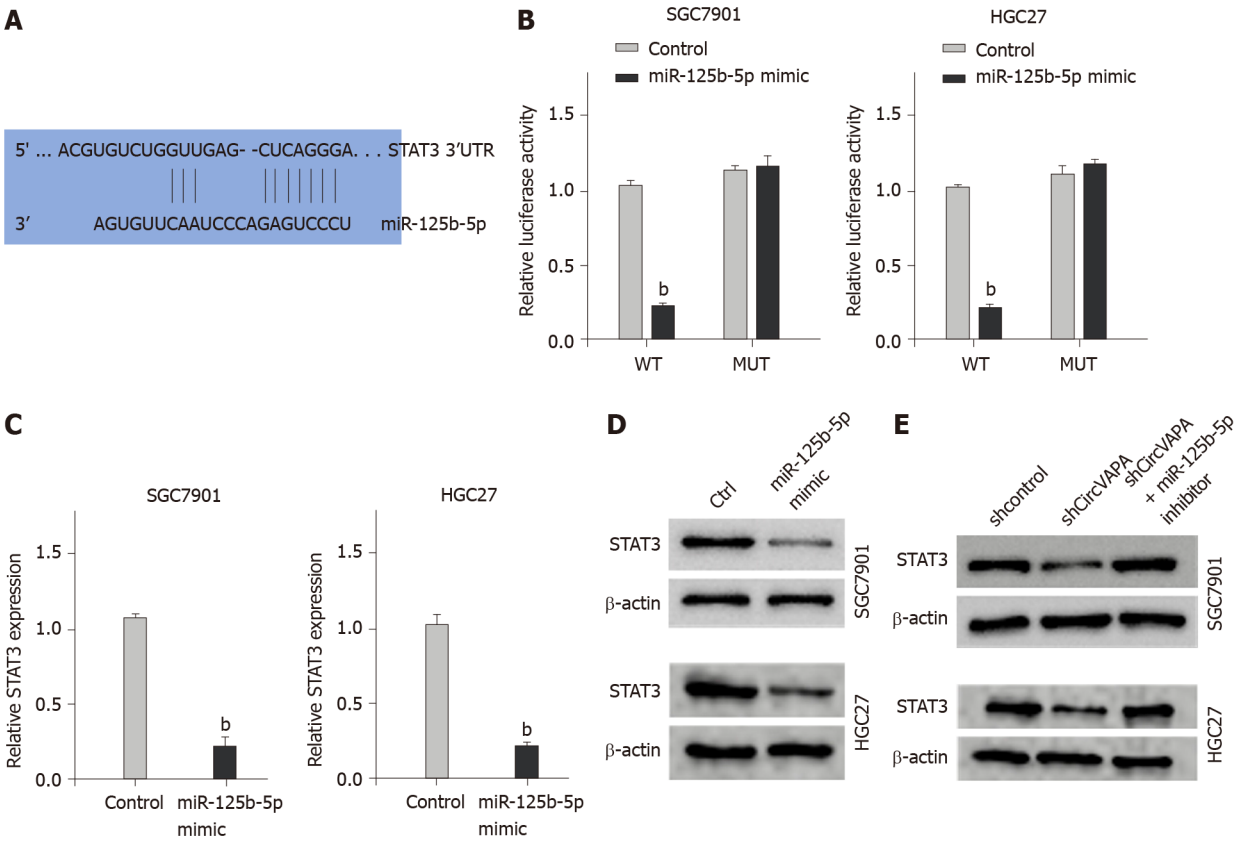

Figure 5 MiR-125b-5p targets STAT3 in gastric cancer cells. A: The binding of miR-125b-5p and the 3' untranslated region of *STAT3* was identified by bioinformatic analysis based on Targetscan (http://www.targetscan.org/vert_72/); B-D: SGC7901 and HGC27 cells were treated with control mimic or miR-125b-5p mimic. The luciferase activities of wild type STAT3 and STAT3 with the miR-125b-5p-binding site mutant were examined by luciferase reporter gene assay (B), the mRNA expression of STAT3 was analyzed by qPCR assay (C), and the protein expression of STAT3 was determined by Western blot analysis (D); E: SGC7901 and HGC27 cells were treated with control shRNA or circVAPA shRNA, or co-treated with circVAPA shRNA and miR-125b-5p inhibitor. The protein expression of STAT3 was tested by Western blot analysis. Data are presented as the mean ± SD. $^{b}P < 0.01$. MUT: Mutant; WT: Wild type.

CONCLUSION

CircVAPA promotes chemotherapy resistance and the malignant progression in GC by modulating miR-125b-5p/STAT3 signaling. Our findings present novel insights into the mechanism by which circVAPA regulates chemotherapy resistance of GC cells. CircVAPA and miR-125b-5p may be considered as potential targets for GC therapy.

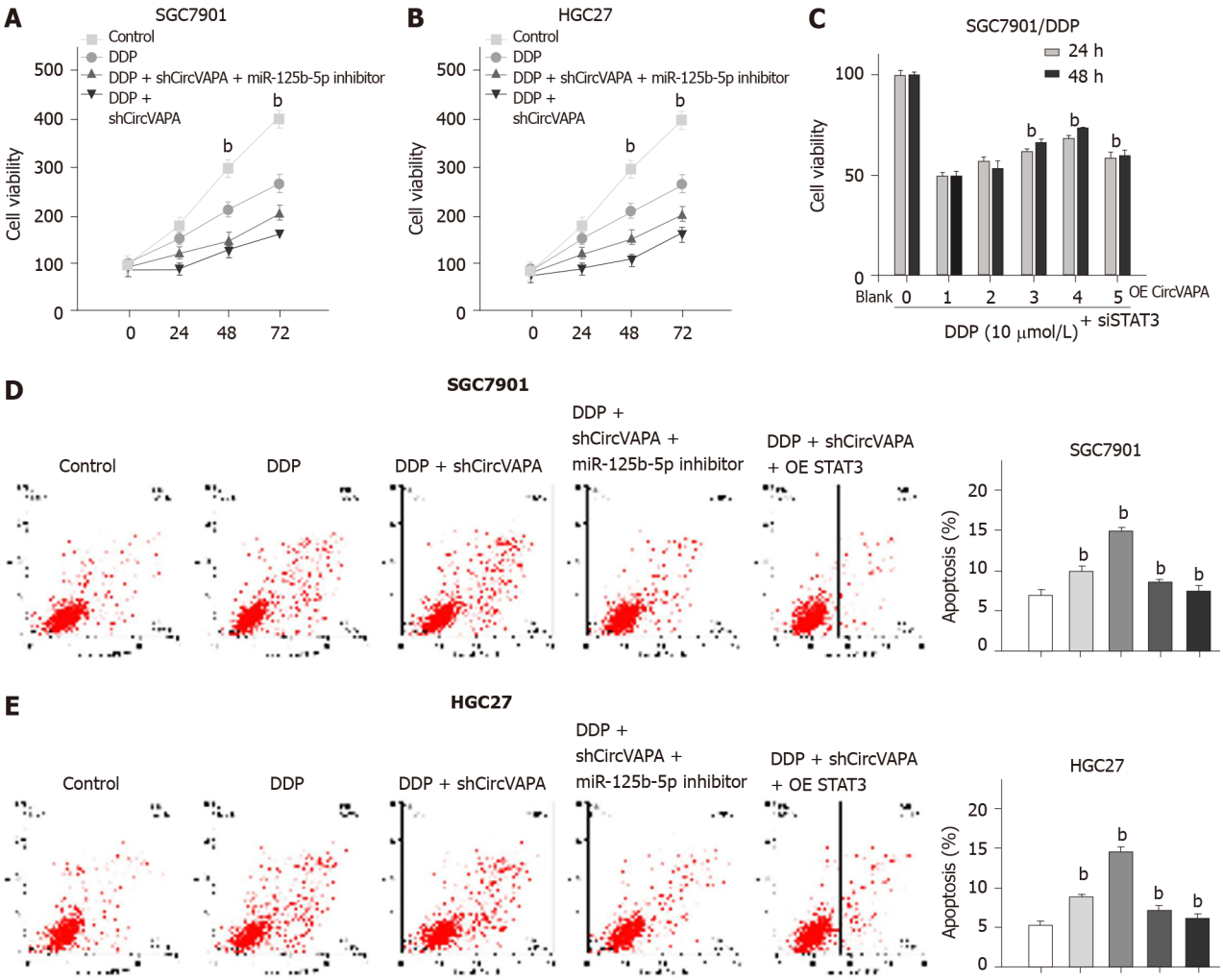

Figure 6 CircVAPA promotes chemotherapy drug resistance of gastric cancer cells by modulating miR-125b-5p/STAT3 axis. A and B: SGC7901 and HGC27 cells were treated with cisplatin (DDP), DDP, and circVAPA shRNA, or co-treated with DDP, circVAPA shRNA, and miR-125b-5p inhibitor. Cell viability was measured by MTT assay; C: SGC7901/DDP cells were treated with DDP or co-treated with DDP and pcDNA3.1-circVAPA at the indicated dose, or co-treated with DDP, pcDNA3.1-circVAPA, and STAT3 siRNA. Cell viability was measured by MTT assay; D and E: SGC7901 and HGC27 cells were treated with DDP, DDP, and circVAPA shRNA, or co-treated with DDP, circVAPA shRNA, and miR-125b-5p inhibitor or pcDNA3.1-STAT3. Cell apoptosis was analyzed by flow cytometry. Data are presented as the mean ± SD. $^{b}P < 0.01$. DDP: Cisplatin.

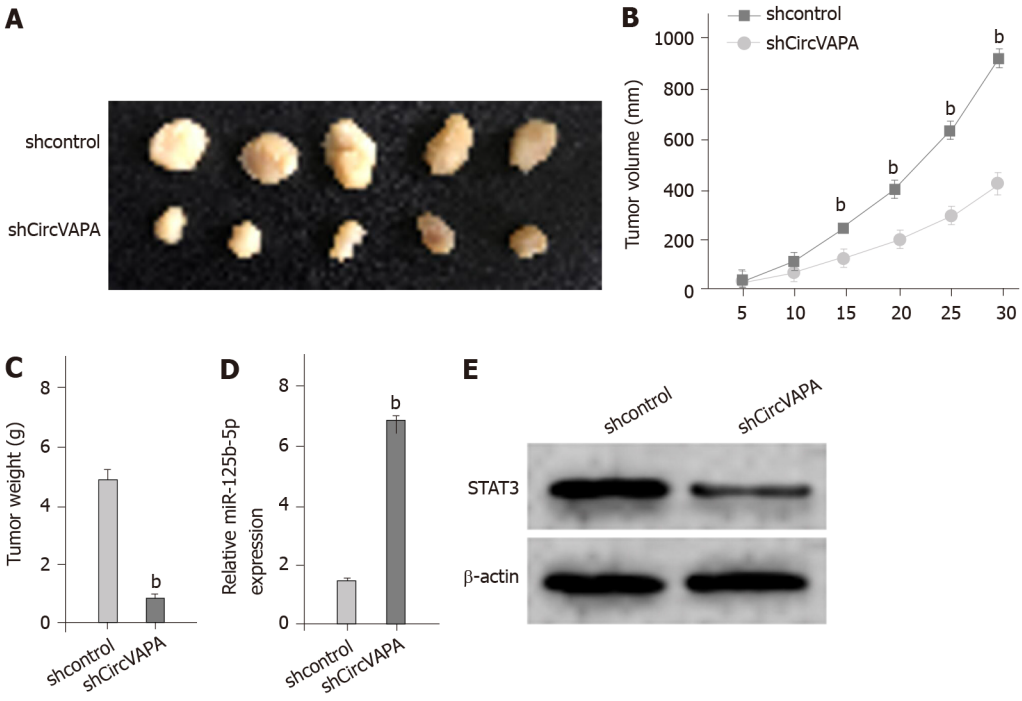

Figure 7 CircVAPA promotes tumor growth of chemoresistant gastric cancer cells *in vivo*. A-D: The impact of circVAPA on tumor growth of melanoma cells *in vivo* was analyzed by nude mice tumorigenicity assay (n = 5). SGC7901/cisplatin cells were treated with control shRNA or circVAPA shRNA. Representative images of dissected tumors from nude mice are shown (A). The average tumor volume (B) and weight (C) were calculated and presented. The expression of miR-125b-5p was measured by qPCR in the tumor tissues of the mice (D); E: The expression of STAT3 was tested by Western blot analysis in the tumor tissues of the mice. Data are presented as the mean ± SD. $^{b}P < 0.01$.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is a common malignant tumor and causes a high incidence of cancer-associated death. Cisplatin (DDP)-based chemotherapy is the main therapy for clinical GC treatment, but the mechanism of chemotherapy resistance is still poorly understood, which is a severe clinical challenge. CircRNAs have been identified to play a vital role in regulating the chemoresistance of gastric cancer cells. The development process of GC chemotherapy resistance is not well-understood. In addition, it is well known that circRNAs and miRNAs are involved in chemotherapy resistance of GC cells. However, the role of circVAPA and miR-125b-5p in regulating the chemotherapy resistance of GC cells has not been reported yet.

Research motivation

To investigate the impact of circVAPA on chemotherapy resistance during the progression of GC.

Research objectives

We collected the clinical GC tissue samples (n = 50) and the adjacent normal tissues (n = 50) and analyzed the effect of circVAPA on GC progression and chemotherapy resistance.

Research methods

The role of circVAPA in chemotherapy resistance and GC progression was analyzed by transwell assay, MTT assay, colony formation assay, healing assay, and wound flow cytometry analysis in GC cells and DDP resistant GC cell lines, and *in vivo* tumorigenicity analysis in nude mice. The mechanism was investigated by quantitative real-time PCR, luciferase reporter assay, and Western blot analysis.

Research results

CircVAPA depletion inhibited proliferation, migration, and invasion and increased apoptosis of GC cells. CircVAPA knockdown reduced tumor growth of GC cells *in vivo*. CircVAPA promoted the chemotherapy resistance of GC cells. We found that

circVAPA targeted miR-125b-5p and miR-125b-5p to STAT3 in GC cells. MiR-125b-5p inhibitor rescued the depletion of circVAPA and enhanced the inhibitory effect of DDP on GC cells, while STAT3 knockdown prevented the proliferation of DDP-treated SGC7901/DDP cells induced by circVAPA overexpression. Depletion of STAT3 and miR-125b-5p inhibitor reversed circVAPA depletion-induced GC cell apoptosis.

Research conclusions

CircVAPA promotes chemotherapy resistance and the malignant progression of GC cells by modulating miR-125b-5p/STAT3 signaling. CircVAPA and miR-125b-5p may be considered as potential targets for GC therapy.

Research perspectives

CircRNA circVAPA promotes chemotherapy drug resistance in the progression of gastric cancer by regulating the miR-125b-5p/STAT3 axis.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Validation of serum tumor biomarkers in predicting advanced cystic mucinous neoplasm of the pancreas

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Author contributions: Sun LQ, Peng LS, and Guo JF contributed equally to this study and should be regarded as co-first authors; Sun LQ, Peng LS, and Guo JF were responsible for study design/planning, study conduct, data analysis, and writing and revising the paper; Jiang F and Cui F assisted with data collection and analysis; Jin ZD and Huang HJ designed the study and they shared co-corresponding authorship; all authors have read and approved the final manuscript.

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Abstract

BACKGROUND

Early detection of advanced cystic mucinous neoplasms [(A-cMNs), defined as high-grade dysplasia or malignancy] of the pancreas is of great significance. As a simple and feasible detection method, serum tumor markers (STMs) may be used to predict advanced intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs). However, there are few studies on the usefulness of STMs other than carbohydrate antigen (CA) 19-9 for early detection of A-cMNs.

AIM

To study the ability of five STMs-CA19-9, carcinoembryonic antigen (CEA), CA125, CA724, and CA242 to predict A-cMNs and distinguish IPMNs and MCNs.

METHODS

We mainly measured the levels of each STM in patients pathologically diagnosed with cMNs. The mean levels of STMs and the number of A-cMN subjects with a higher STM level than the cutoff were compared respectively to identify the ability of STMs to predict A-cMNs and distinguish MCNs from IPMNs. A receiver operating characteristic curve with the area under curve (AUC) was also created to identify the performance of the five STMs.

RESULTS

A total of 187 patients with cMNs were identified and 72 of them showed A-cMNs. We found that CA19-9 exhibited the highest sensitivity (SE) (54.2%) and accuracy (76.5%) and a moderate ability (AUC = 0.766) to predict A-cMNs. In predicting high-grade dysplasia IPMNs, the SE of CA19-9 decreased to 38.5%. The ability of CEA, CA125, and CA724 to predict A-cMNs was low (AUC = 0.651, 0.583, and 0.618, respectively). The predictive ability of CA242 was not identified. The combination of STMs improved the SE to 62.5%. CA125 may be specific to the

interest.

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diagnosis of advanced MCNs.

CONCLUSION
CA19-9 has a moderate ability, and CEA, CA125, and CA724 have a low ability to predict A-cMNs. The combination of STM testing could improve SE in predicting A-cMNs.

Key Words: Serum tumor markers; Diagnosis; Advanced cystic mucinous neoplasms; Mucinous cystic neoplasms; Intraductal papillary mucinous neoplasms

Core Tip: The value of serum tumor markers (STMs) in predicting advanced cystic mucinous neoplasms (A-cMNs) has not been well studied except for carbohydrate antigen (CA) 19-9. Our study illustrates for the first time the potential value of multiple STMs in the diagnosis of A-cMNs. CA19-9 was the most accurate STM in predicting A-cMNs with a moderate ability. CEA, CA125, and CA724 showed a low ability to predict A-cMNs. CA125 may be specific to the diagnosis of advanced mucinous cystic neoplasms. CA242 was not identified as a useful STM in predicting A-cMNs in our study. The combination of STMs could improve sensitivity in predicting A-cMNs.

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INTRODUCTION

Pancreatic cystic lesions (PCLs) are being detected more frequently due to advancements in radiological technologies. Their prevalence ranges from 1.9% to 49.1% in different races[1-3]. The primary management goal of PCLs is to correctly identify malignant and invasive PCLs. Serum tumor markers (STMs) may play an adjunct role to imaging features in the evaluation of PCLs.

Cystic mucinous neoplasms (cMNs) of the pancreas, including mucinous cystic neoplasms (MCNs) and intraductal papillary mucinous neoplasms (IPMNs), have the potential to undergo malignant transformation, which is a cause for concern. Early detection of cMNs with high-grade dysplasia or invasive cancer [advanced cMNs (A-cMNs)] is important for improving prognoses.

MCN and IPMN have different biological behaviors and originate from different embryonic layers[4]. Although they may have similar imaging presentations, especially branch duct IPMN and MCN, they are different tumor types. Theoretically, they may also show different specific STMs. However, there is little available data demonstrating the difference.

STM testing is accessible in routine health checkups. It is more suitable as a screening modality than cross-imaging technologies. Several STMs, including carbohydrate antigen (CA) 19-9, carcinoembryonic antigen (CEA), CA125, CA724, and CA242, have been reported to be associated with the presence, metastasis, and recurrence of pancreatic adenocarcinoma[5-9]. However, the value of these STMs for PCLs is not well studied except for CA19-9 and CEA. CA19-9 is considered an independent predictor of malignancy in PCLs by many guidelines[10-12]. The value of CEA is mostly based on evidence of cyst fluid analyses, and it has the ability to distinguish mucinous from non-mucinous PCLs[13]. The sensitivity (SE) of CEA in serum is considered relatively low in predicting advanced IPMNs[14-16]. No previous studies have reported the value of CA125, CA724, or CA242 in predicting A-cMNs.

To evaluate the ability of CA19-9, CEA, CA125, CA724, and CA242 in predicting A-cMNs and whether the STM levels vary in MCNs and IPMNs, we conducted a retrospective cohort study of 187 patients diagnosed with PCLs and assessed their STM levels. Although we have obtained some promising findings, as a retrospective

study with incomplete data, these findings need to be further confirmed by multi-center prospective studies.

MATERIALS AND METHODS

Patients

We conducted a retrospective study at Changhai Hospital affiliated to Second Military Medical University, one of the largest pancreatic disease research centers in Shanghai, China. Patients who underwent surgical resection for an identified pancreatic cyst and whose final pathological outcomes were identified with cMNs were included in our study. A-cMNs were defined as MCNs and IPMNs with high-grade dysplasia or malignancy identified by pathological outcomes. Patient demographics, pathologic data, and STMs were extracted. The Institutional Review Board of Changhai Hospital approved the study.

The decision regarding surgical treatment was made by our multidisciplinary hepato-pancreatobiliary team for the vast majority of patients according to the FG guidelines of 2006 and 2012[17,18]; the decisions for the remaining cases were made according to the patients' own wishes.

Serum tumor marker analyses

Serum CA19-9, CA125, CA724, CA242, and CEA were measured by using electrochemiluminescence immunoassays on a Roche Cobas E601 immunoassay analyzer (Roche Diagnostics, Mannheim, Germany) equipped with dedicated reagents, according to the manufacturer's instructions. All of the assays were carried out at the Department of Laboratory Medicine, Changhai Hospital.

In our center, the cutoff values for STMs were: CA19-9, 37.0 U/mL; CEA, 5.0 ng/mL; CA125, 35.0 U/mL; CA724, 9.8 U/mL; and CA242, 20.0 U/mL. The mean levels of STMs and the number of A-cMN subjects with a higher STM level than the cutoff were compared to identify the ability of STMs to predict A-cMNs. STMs with a significant difference between A-cMNs and non-A-cMNs for both the mean level and number of subjects were considered to have the ability to predict A-cMNs. A receiver operating characteristic curve was created to identify the true ability of these STMs in predicting A-cMNs. STMs with areas under curve (AUCs) higher than 0.9, 0.7-0.9, and lower than 0.7 were considered to have strong, moderate, and low abilities in predicting A-cMNs, respectively. The SE, specificity (SP), and accuracy (AC) were also calculated to measure performance of the five STMs. For combination analysis, if any of these STM levels were elevated (higher than the cutoff level) in one subject, the combination was considered positive.

Statistical analysis

Categorical data were reported as frequencies or percentages. Continuous data are reported as the mean ± SD. Two-tailed Student's *t* tests for continuous variables and chi-square tests for categorical variables were used to identify predictors of A-cMNs. The *Z* test was calculated to determine whether the AUCs are significantly different between STMs. $P < 0.05$ was considered statistically significant. Data were analyzed with IBM Statistic Package for Social Science Statistics version 22.0 (IBM Corp., Armonk, NY, United States).

RESULTS

Patient selection and characteristics

From January 2013 to June 2019, 187 patients were identified with cMNs by surgical resection specimens in our center and were included in our study. Of the 187 patients, 151 (80.7%) had IPMNs, and 36 (19.3%) had MCNs. Of the 151 IPMN subjects, 46 IPMNs with invasive cancer and 13 IPMNs with high-grade dysplasia were identified. Of the 36 MCN subjects, 12 MCNs with invasive cancer and one MCN with high-grade dysplasia (HGD) were identified. In total, 72 advanced-cMNs were included in our study. The mean age of the overall study cohort was 60.2 ± 11.9 years, and males (109, 58.3%) were slightly more predominant than females (78, 41.7%). The median cyst size (interquartile range) of the study cohort was 28.5 mm (19-42 mm). The number of patients with cysts located in the pancreatic head/body/tail/whole pancreas was 111, 31, 30, and 15, respectively (Table 1). Male sex ($P = 0.001$), aging ($P = 0.014$), and large

Table 1 Baseline characteristics and performance of each serum tumor maker in predicting advanced cystic mucinous neoplasms

	Overall	Benign	Advanced	*P* value[1]
Patients characteristics (*n* = 187)				
Male (*n*, %)	109 (58.3)	63 (54.8)	46 (63.9)	0.007
Age (Median, IQR)	62 (54-68)	61 (51-68)	63.5 (56-70)	0.014
Cyst size (Median, IQR, mm)	28.5 (19-42)	24.5 (15-37)	38.5 (28-59.5)	< 0.0001
Location (H/B/T/D)	111/31/30/15	63/20/20/10	48/11/10/5	
CA19-9 (*n* = 187)				
Median, IQR (U/mL)	10.11(4.4-52.9)	7.1(3.4-16.3)	55.5 (9.3-319.3)	< 0.0001
≥ 37/< 37 U/mL	50/137	11/104	39/33	< 0.0001
CEA (*n* = 187)				
Median, IQR (ng/mL)	2.37 (1.54-3.98)	2.23 (1.5-3.3)	3.1 (2.02-5.2)	< 0.0001
≥ 5/< 5 ng/mL	27/160	9/106	18/54	0.001
CA125 (*n* = 135)				
Median, IQR (U/mL)	12.2 (8.2-18.8)	12.1 (7.0-16.8)	12.75 (8.9-23.4)	0.002
≥ 35 /< 35 U/mL	10/125	1/82	9/43	0.002
CA724 (*n* = 116)				
Median, IQR (U/mL)	1.6 (1.025-3.15)	1.44 (1.0-2.66)	2.4 (1.07-6.8)	0.014
≥ 9.8/< 9.8 U /mL	10/106	3/70	7/36	0.05
CA242 (*n* = 31)				
Median, IQR (U/mL)	7.13 (3.6-12.7)	5.35 (2.7-7.9)	11.1 (6.95-31.7)	0.136
≥ 20/< 20 U/mL	6/25	1/15	5/10	0.08

[1]*P* values were based on student's *t* test comparing mean values of each serum tumor maker. cMN: Cystic mucinous neoplasm; IQR: Interquartile range; CEA: carcinoembryonic antigen; CA: Carbohydrate antigen.

cyst size ($P < 0.0001$) were associated with the malignant transformation of MNs.

Performance of each STM in predicting advanced cystic mucinous neoplasms

Receiver operating characteristic curves are demonstrated in Figure 1. The baseline characteristics and performance of each STM, including the overall characteristics and the classification of benign *vs* malignant by surgical pathology, are shown in Table 1. The SE, SP, positive predictive value, negative predictive value, AC, and AUC of each STM and their combination in predicting A-cMNs are presented in Table 2.

Serum CA19-9 tests were available for all 187 subjects. The mean CA19-9 level was 121.3 ± 10.11 U/mL, and CA19-9 ≥ 37 U/mL was observed in 50 subjects, including 39 advanced cases. Serum CEA tests were also available for all 187 subjects. The mean CEA level was 3.46 ± 3.75 ng/mL, and CEA ≥ 5 ng/mL was observed in 27 subjects, including 18 advanced cases. Serum CA125 tests were available for 135 (72.2%) subjects, and the mean level was 17.65 ± 21.56 U/mL. An elevated CA125 level ≥ 35 U/mL was observed in ten subjects, including nine advanced cases. Serum CA724 tests were available for 116 (62%) subjects, and the mean level was 5.46 ± 17.56 U/mL. An elevated CA724 level ≥ 9.8 U/mL was observed in ten subjects, including seven advanced cases. Serum CA242 tests were available for 31 (16.6%) subjects, and the mean level was 32.12 ± 92.2 U/mL. An elevated CA242 Level ≥ 20 U/mL was observed in six subjects, including five advanced cases (Table 1).

CA19-9 (both $P < 0.0001$), CEA ($P < 0.0001$ and 0.001), CA125 (both $P = 0.002$), and CA724 ($P = 0.014$ and 0.05) all had the ability to predict A-cMNs because they showed significant differences in mean levels between the benign and advanced groups and in the number of advanced cases with a higher STM level than the cutoff (Table 1). These STMs had relatively high SP (90.4%, 92.17%, 98.8%, and 95.9%). However, their SE was suboptimal (54.2%, 25%, 17.3%, and 16.3%) (Table 2). Comparing their AUCs, CA19-9

Table 2 Performance of five serum tumor makers in predicting advanced cystic mucinous neoplasms

	SE (95%CI)	SP (95%CI)	PPV (95%CI)	NPV (95%CI)	AC	AUC	*P* value
CA19-9 (%)	54.2 (42.1-65.8)	90.4 (83.2-94.5)	78 (63.6.7-88)	75.9 (67.7-82.6)	76.5	0.766	
CEA (%)	25 (15.9-45.9)	92.17 (85.26-96.13)	66.67 (46-82.8)	66.3 (58.3-73.4)	66.31	0.651	0.04
CA125 (%)	17.3 (8.7-30.8)	98.8 (92.54-99.9)	90 (54.1-99.5)	65.5 (56.5-73.7)	67.4	0.583	0.004
CA724 (%)	16.3 (7.3-31.3)	95.9 (87.7-98.9)	70 (35.4-91.9)	66 (56.1-74.8)	66.4	0.618	0.03
CA242 (%)	31.25 (12.1-58.5)	93.3 (66-99.7)	83.3 (36.5-99.1)	56 (35.3-75)	61.3	0.758	0.81
CA199 ± any one of other STMs	62.5 (50.3-73.4)	83.5 (75.1-89.5)	70.3 (57.4-80.8)	78 (69.5-84.8)	75.4	0.715	0.32

P value: Comparison results of areas under curves for carbohydrate antigen 19-9 and other serum tumor makers in predicting advanced-cystic mucinous neoplasms. SE: Sensitivity; SP: Specificity; PPV: Positive predictive value; NPV: Negative predictive value. AC: Accuracy; AUC: Area under curve; CA: Carbohydrate antigen; STM: Serum tumor maker.

had a significantly better performance (0.766) than CEA (0.651, $P = 0.04$), CA125 (0.583, $P = 0.004$), and CA724 (0.618, $P = 0.03$). CEA, CA125, and CA724 had comparable performances in predicting A-cMNs ($P > 0.05$ for all) (Table 3).

The ability of CA242 to predict A-cMNs was not identified by our study. We found no significantly higher mean level nor any significantly greater number of subjects with a higher STM level than the cutoff in the advanced groups ($P = 0.136$ and 0.08) despite the comparable AUC to CA19-9 (0.758, $P = 0.81$). The combination of STMs increased the SE to 62.5%, but the SP decreased to 83.5%. However, the diagnostic AC remained high (75.4%) (Table 2). The AUC was comparable to that of CA19-9 alone ($P = 0.32$).

Performance of STMs in predicting advanced IPMNs and MCNs

In all 151 IPMN subjects, both CA19-9 and CEA were available. CA125, CA724, and CA242 were available in 109, 96, and 28 subjects, respectively. CA19-9, CEA, and CA724 were identified as having definite abilities in predicting advanced IPMNs (Table 4).

In all 36 MCN subjects, both CA19-9 and CEA were available. CA125, CA724, and CA242 were available in 26, 20, and 3 subjects, respectively. CA19-9, CEA, and CA125 were identified as having definite abilities in predicting advanced MCNs. Moreover, the subjects with advanced MCN were much older than those with non-advanced MCN ($P = 0.005$) (Table 5).

The mean level and elevated number of subjects for CA19-9, CEA, CA724, and CA242 were not significantly different between advanced MCNs and IPMNs. However, the mean CA125 Level in advanced MCNs was 74.4 ± 60.3 U/mL, which was much higher than that in advanced IPMNs (17.3 ± 16.4 U/mL, $P < 0.0001$). CA125 tests were available in 7 of 13 advanced MCNs, and five (71.4%) subjects had elevated CA125 Levels. Meanwhile, CA125 tests were available in 45 of 59 advanced IPMNs, and four (8.9%) subjects had elevated CA125 levels. The number of subjects with elevated CA125 levels in advanced MCNs was also higher than that in IPMNs ($P < 0.0001$) (Table 6). The SE, SP, AC, and AUC of the standard serum CA125 cutoff level (35 U/L) in differentiating advanced MCNs and advanced IPMNs were 71.4%, 91.1%, 88.5%, and 0.779, respectively. Therefore, in addition to the ability to predict A-cMNs, serum CA125 testing may be useful to differentiate advanced MCNs and advanced IPMNs.

Performance of each STM in predicting high-grade dysplasia IPMN

Thirteen cases of IPMN and one case of MCN with high-grade dysplasia were identified in our study. Due to the small number of MCNs with HGD, we only assessed the performance of STMs in predicting IPMN with HGD. CA19-9 and CEA were both available in all 13 HGD IPMNs. The mean levels of CA19-9 and CEA were 182.5 ± 356.5 U/mL and 3.77 ± 2.17 ng/mL for HGD IPMNs, respectively. There were five cases with CA19-9 levels > 37 U/mL and three cases with CEA levels > 5 ng/mL. In addition, seven patients with HGD IPMNs were tested for CA125 (mean level 10.9 ± 2.17 U/mL), five for CA724 (mean level 3.09 ± 2.4 U/mL), and three for CA242 (mean level 172.7 ± 283.5 U/mL). Comparison of levels of the five STMs between benign and HGD IPMN patients showed that only CA19-9 had the ability to predict HGD IPMNs.

Table 3 Receiver operating characteristic curve comparison analyses of each serum tumor marker

ROC curve	ΔAUC[1]	Standard error	*Z* statistic	*P* value
CA19-9 *vs* CEA	0.115	0.038 and 0.042	2.03	0.04
CA19-9 *vs* CA125	0.183	0.038 and 0.051	2.88	0.004
CA19-9 *vs* CA724	0.148	0.038 and 0.058	2.14	0.03
CA19-9 *vs* CA242	0.008	0.038 and 0.090	0.08	0.81
CEA *vs* CA125	0.068	0.042 and 0.051	1.03	0.30
CEA *vs* CA724	0.033	0.042 and 0.058	0.46	0.62
CA125 *vs* CA724	0.035	0.051 and 0.058	0.45	0.63

[1]ΔAUC: The gap between the two areas under the curves. CA: Carbohydrate antigen; CEA: Carcinoembryonic antigen; ROC: Receiver operating characteristic; AUC: Area under curve.

Table 4 Performance of each serum tumor maker in predicting advanced intraductal papillary mucinous neoplasms

	Overall	Benign	Advanced	*P* value[1]
CA19-9 (*n* = 151)				
Median, IQR (U/mL)	9.7 (3.75-46.3)	6.05 (3.1-15.2)	46.3 (9.0-166.4)	< 0.0001
≥ 37/< 37 U/mL	40/111	9/83	31/8	< 0.0001
CEA (*n* = 151)				
Median, IQR (ng/mL)	2.7 (1.85-4.16)	2.46 (1.67-3.44)	3.1 (2.05-5.3)	0.002
≥ 5/< 5 ng/mL	24/127	9/83	15/44	0.01
CA125 (*n* = 109)				
Median, IQR (U/mL)	11.9 (8.0-17.35)	11.65 (6.8-16.6)	12.1 (8.7-18.8)	0.051
≥ 35 /< 35 U/mL	5/104	1/63	4/41	0.182
CA724 (*n* = 96)				
Median, IQR (U/mL)	1.64 (1.04-3.17)	1.44 (1.02-2.7)	2.5 (1.16-6.8)	0.022
≥ 9.8/< 9.8 U /mL	9/87	2/56	7/31	0.035
CA242 (*n* = 28)				
Median, IQR (U/mL)	7.44 (4.1-12.7)	5.7 (3.6-8.23)	9.8 (6.9-20.7)	0.174
≥ 20/< 20 U/mL	5/23	1/12	4/11	0.416

[1]*P* values were based on student's *t* test comparing mean values of each serum tumor maker. CA: Carbohydrate antigen; CEA: Carcinoembryonic antigen; IQR: Interquartile range.

The SE, SP, AC, and AUC values of CA199 for differentiating HGD IPMNs and benign IPMNs were 38.5%, 90.2%, 83.8%, and 0.643, respectively.

DISCUSSION

Due to the absence of reliable standards for distinguishing advanced PCLs from non-advanced PCLs, the clinical management of PCLs remains difficult. PCLs, especially cMNs, have the potential to undergo carcinogenesis, and malignant transformation may take a relatively long time[3]. Therefore, early detection of A-cMNs is key to the management of PCLs. Current guidelines recommend long-term, routine yearly surveillance by magnetic resonance imaging (MRI)/magnetic resonance cholangiopancreatography (MRCP), and/or endoscopic ultrasound (EUS)[10-12]. The benefit of

Table 5 Performance of each serum tumor maker in predicting advanced mucinous cystic neoplasms

	Overall	Benign	Advanced	*P* value[1]
CA19-9 (*n* = 31)				
Median, IQR (U/mL)	14.7 (7.3-108.1)	7.73 (5.63-24.2)	157 (15.7-1100)	0.001
≥ 37/< 37 U/mL	10/26	2/21	8/5	0.003
CEA (*n* = 31)				
Median, IQR (ng/mL)	1.5 (1.05-2.58)	1.5 (1.0-1.87)	2.56 (1.31-5.57)	0.004
≥ 5/< 5 ng/mL	3/33	0/23	3/10	0.04
CA125 (*n* = 26)				
Median, IQR (U/mL)	14.5 (10.0-25.7)	14.4 (9.2-20.1)	68.6 (11.9-125)	< 0.0001
≥ 35 /< 35 U/mL	5/21	0/19	5/2	< 0.0001
CA724 (*n* = 20)				
Median, IQR (U/mL)	1.5 (0.96-2.7)	1.52 (0.99-2.24)	1.53 (0.8-6.8)	0.538
≥ 9.8/< 9.8 U /mL	1/19	1/14	0/5	1.00
CA242 (*n* = 3)				
Median, IQR (U/mL)	2.39 (0.1)	1.25 (0.1)	35.35	Not applicable
≥ 20/< 20 U/mL	1/2	0/2	1/0	0.333

[1]*P* values were based on student's *t* test comparing mean values of each serum tumor maker. CA: Carbohydrate antigen; CEA: Carcinoembryonic antigen; IQR: Interquartile range.

Table 6 Comparison of serum carbohydrate antigen 125 levels in mucinous cystic neoplasms and intraductal papillary mucinous neoplasms

	Overall	Benign	Advanced
mean ± SD (U/mL) (IPMN)	14.6 ± 12.2	12.7 ± 7.56	17.3 ± 16.4
mean ± SD (U/mL) (MCN)	30.5 ± 40.5	14.3 ± 6.4	74.4 ± 60.3
Comparison *P* value	0.001	0.69	< 0.0001
≥ 35/< 35 U/mL (IPMN)	5/104	1/63	4/41
≥ 35/< 35 U/mL (MCN)	5/21	0/19	5/2
Comparison *P* value	0.03	1.00	< 0.0001

MCN: Mucinous cystic neoplasm; SD: Standard deviation; IPMN: Intraductal papillary mucinous neoplasm.

yearly surveillance in reducing pancreatic cancer-related mortality remains to be established. Further, routine surveillance may impose psychological and economic burdens[19]. STMs are much more accessible than MRI/MRCP/EUS, which can be obtained during health checkups. The value of STMs in predicting advanced PCLs remains to be fully elucidated.

The role of STMs in the early detection of pancreatic ductal adenocarcinoma (PDA) is doubtful due to their low SE. However, as a tumor progresses, the level of STMs increases[14]. Moreover, the 5-year survival rate after surgical resection of A-MNS was much higher than that of PDA[20-23]. In other words, although STMs may not be able to detect early A-MNs, they can contribute to finding late A-MNs that are more indolent than PDA, and the prognosis is still satisfactory[24]. Thus, unlike the role of STMs in PDA, STM testing may play a more active role in the management of PCLs.

CA19-9 was widely acknowledged as the most accurate STM for predicting A-cMNs, and the value of CA19-9 was also identified in our study. Although false negative results and false positive results (caused by biliary tract obstruction and inflammation, pancreatitis, and other digestive cancers)[9] limit its clinical application,

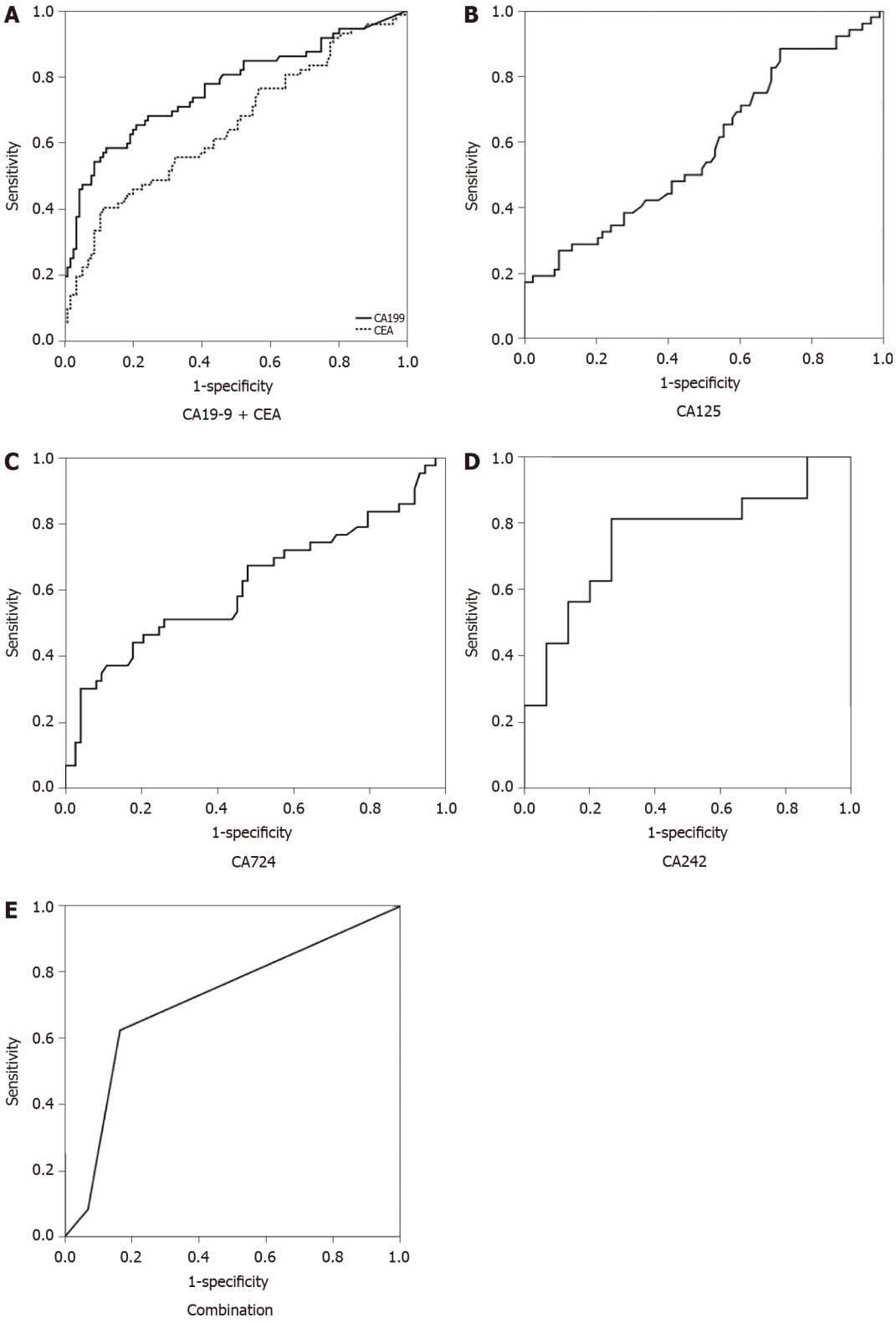

Figure 1 Receiver operating characteristic curves. A: Carbohydrate antigen (CA) 19-9 + carcinoembryonic antigen; B: CA125; C: CA724; D: CA242; and E: Their combination in the diagnosis of advanced cystic mucinous neoplasms of the pancreas.

it is still widely used in the management of PCLs. The pooled SE for CA19-9 in predicting advanced PCLs was approximately 40% by a meta-analysis[14]. As our study showed, although the best performances were observed in all analysis groups, the SE was suboptimal, and the AUC for CA19-9 was 0.766, which indicated that the ability of CA19-9 to predict A-cMNs was moderate. Moreover, if merely based on the commonly used cutoff (37 U/mL), up to 33/72 (45.8%) malignant subjects would be considered negative, and 11/115 (9.6%) benign subjects would be considered positive.

Furthermore, we for the first time report that CA19-9 was still a useful diagnostic tool for predicting HGD IPMNs, but the SE and AUC decreased as expected.

The reported SE and SP of CEA for IPMNs were 18% and 93%, respectively[14]. In our study, serum CEA detection had the ability to distinguish A-cMNs from non-A-cMNs. However, the AUC for CEA was 0.651, and the SE was 25%. Both indicators performed worse than CA19-9. The results indicated that the predictive value of CEA for A-cMNs was low. Therefore, it is essential to explore other biomarkers to supplement CA19-9 and CEA to improve the diagnosis of A-cMNs.

Our study identified CA125 and CA724 as useful STMs in predicting A-cMNs with a relatively low SE (17.3% and 16.3%) and low AUC (0.583 and 0.618), which indicated that both STMs were weak predictors of A-cMNs. Therefore, CA125 and CA724 could not be identified as valuable screening markers for A-cMNs alone despite their relatively high SP (98.8% and 95.9%). Nevertheless, we identified that serum CA125 was more likely a predictor of advanced MCN than advanced IPMN. The results were similar to those of two recently published studies[25,26]. The possible reason may be the tissue histogenesis of MCN. Elevated CA125 levels are common in serous ovarian tumors. However, for mucinous ovarian tumors, an increase in serum CA125 levels is not uncommon[27]. MCNs and mucinous ovarian tumors both originate from primordial germ cells[4], and ovarian-like stroma can be obtained from the tissue of advanced MCNs[28]; thus, advanced MCNs may theoretically have an increased CA125 level as well as ovarian tumors. If a patient's serum CA125 level is elevated, it may be necessary to examine the pancreas to exclude advanced MCNs after an ovarian examination. However, the result was concluded from a small sample size, and the true value of CA125 in the management of A-cMNs needs to be further studied.

We did not find that CA242 showed predictive value. This may be due to our study's small sample size. The recognition of CA242 has not been established until recent years in our hospital. As a result, there were fewer patients who underwent CA242 testing in our hospital than those who were tested for other STMs. However, CA242 had a comparable AUC to CA19-9 (0.758 *vs* 0.766, $P = 0.81$). The value of CA242 in PCLs may be verified by enlarging the sample size.

The combination of multiple STMs can improve their diagnostic AC in pancreatic cancer[5]. However, no one has explored this field in PCLs. In our study, the combination of STMs slightly increased the SE of CA19-9 to 62.5%. However, the SP decreased to 83.5%, and the AUC decreased to 0.715. The bias may exist due to the incomplete data for CA125, CA724, and CA242. Overall, the more data from these incomplete STMs that were included in our study, the more positive subjects were associated with combined results, and the SE was improved. Since STMs are the modalities for screening, the high SE of the combination will lead to more subjects undergoing further imaging evaluation to avoid missed diagnosis of advanced cases, which we deem justified. In addition, the reported AC of CT for identifying benign from malignant cysts was 71%–80%, and the AC of MRI or MRCP for differentiating a benign from a malignant cyst ranged from 55% to 76%[12]. The diagnostic accuracies were very similar to the AC of STMs that we reported in this research. Although imaging modalities can provide more information, we still believe that as a screening tool, serum STM detection may play a role in the management of PCLs.

Our study has limitations that merit discussion. First, this work was designed to be a retrospective, single-center study; hence, selection bias could not be completely avoided. Thus, the results of this study might not be applicable to other medical settings. Second, the CA125, CA724, and CA242 data were incomplete, which might influence the statistical results and decrease the AC of the combination analysis. Third, our analysis focused on cMNs. The application of our study might be limited in clinical practice due to the difficulty of making a definitive diagnosis preoperatively. Fourth, due to the lack of data, other STMs associated with pancreatic cancer, such as CA50, were not included.

CONCLUSION

In conclusion, this is the first study to illustrate the potential value of multiple STMs for the diagnosis of A-cMNs. CA19-9 is the most accurate STM for predicting A-cMNs, showing a moderate ability. CEA, CA125, and CA724 present a low ability to predict A-cMNs. CA125 may be specific to the diagnosis of advanced-MCNs. CA242 is not identified as a useful STM for predicting A-cMNs in our study; however, the AUC for CA242 is comparable to that for CA19-9. Thus, a greater sample size will be necessary to identify the true value of CA242. A combination of the five STMs could improve SE

in predicting A-cMNs.

ARTICLE HIGHLIGHTS

Research background

Early detection of advanced cystic mucinous neoplasms (A-cMNs) of the pancreas is key to the management of pancreatic cystic lesions (PCLs) in relation to the carcinogenic potential of cMNs. However, the long-term, routine yearly surveillance by imaging methods recommended by the current guidelines will impose heavy psychological and economic burdens on patients.

Research motivation

As an economical and feasible detection method, serum tumor markers (STMs) can be obtained during ordinary health checkups. However, the role of STMs in predicting advanced PCLs remains elusive. In view of the consistency between the increasing level of STMs and tumor progression, STM detection may play an important role in the early diagnosis of advanced PCLs.

Research objectives

This study aimed to evaluate the ability of five common serum tumor markers to predict A-cMNs separately and in combination. The relevant research results may be beneficial to the management of PCLs and the optimization of guidance or consensus.

Research methods

This retrospective cohort study mainly measured the levels of serum carbohydrate antigen (CA) 19-9, carcinoembryonic antigen (CEA), CA125, CA724, and CA242 in patients pathologically diagnosed with cMNs to identify the ability of these STMs to predict A-cMNs and distinguish mucinous cystic neoplasms (MCNs) from intraductal papillary mucinous neoplasms. A receiver operating characteristic curve with an area under curve and the sensitivity (SE), specificity (SP), and accuracy were also created to identify the performance of the five STMs.

Research results

CA19-9 showed the highest SE and accuracy and a moderate ability to predict A-cMNs. The ability of CEA, CA125, and CA724 to predict A-cMNs was low. The predictive ability of CA242 was not identified. A combination of STMs could improve SE. CA125 was more likely a predictor of advanced MCNs than advanced intraductal papillary mucinous neoplasms.

Research conclusions

CA19-9 showed a moderate ability, and CEA, CA125, and CA724 showed a low ability to predict A-cMNs. Detection of multiple STMs could improve SE in predicting A-cMNs, which has great potential to improve the early diagnosis rate of advanced PCLs in clinical practice. CA125 may be specific to the diagnosis of advanced MCNs and can be used as a reminder for physicians not to ignore pancreatic examination in patients with elevated CA125.

Research perspectives

A multicenter prospective study that monitors PCL patients with detailed data on serum tumor markers should be performed.

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ORIGINAL ARTICLE

Retrospective Study

Washed microbiota transplantation reduces proton pump inhibitor dependency in nonerosive reflux disease

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Author contributions: Zheng YM contributed to the study design, performed the experiments, and wrote the paper; Wu LH contributed to the study design, provided critical revision of the article for important intellectual content, and made final approval of the article; Chen XY, Cai JY, Zhang M, and Xia HHX designed the study and contributed to the analysis and interpretation of the data; Yuan Y, Xie WR, He XX, and Xu JT provided clinical advice and supervised the report.

Institutional review board statement: This retrospective study was approved by the Ethics Committee of Guangdong Pharmaceutical University (Approval No. Yilun Shen[2019] No. 93 01).

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Abstract

BACKGROUND

The pathogenesis of gastroesophageal reflux disease (GERD) is closely associated with the intestinal bacteria composition and their metabolites.

AIM

To investigate whether washed microbiota transplantation (WMT) improves symptoms of nonerosive reflux disease (NERD) with proton pump inhibitor (PPI) dependency.

METHODS

Patients with recurrent NERD and PPI dependency at the First Affiliated Hospital of Guangdong Pharmaceutical University from 2017 to 2018 were included and divided into a WMT or PPI group treated with PPI with/without WMT. The endpoint was NERD symptom frequency evaluated 1 mo after WMT using reflux disease questionnaire (RDQ) and GERD questionnaire (GERDQ) scores, remission time, PPI dose, and the examination of intestinal mucosal barrier function.

RESULTS

In the WMT (n = 15) and PPI (n = 12) groups, the total remission rate at 1 mo after

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treatment was 93.3% *vs* 41.7%. Compared with the PPI group, the WMT group showed better results in GERDQ (P = 0.004) and RDQ (P = 0.003) and in remission months (8 *vs* 2, P = 0.002). The PPI dose was reduced to some extent for 80% of patients in the WMT group and 33.3% in the PPI group. In 24 patients, intestinal mucosal barrier function was examined before treatment, and changes in the degree of damage were observed in 13 of these patients after treatment. Only one of the 15 patients had minor side effects, including a mushy stool two or three times a day, which resolved on their own after 1 wk.

CONCLUSION
This study is the first to demonstrate that WMT may be safe and effective for relieving NERD symptoms and reducing PPI dependency and recurrence.

Key Words: Nonerosive reflux disease; Washed microbiota transplantation; Proton pump inhibitor dependency; Intestinal bacteria; Lipopolysaccharide; Small intestinal bacterial overgrowth

Core Tip: In this study, we demonstrated for the first time that washed microbiota transplantation (WMT) is safe and effective for treating patients with nonerosive reflux disease (NERD) and proton pump inhibitor (PPI) dependency compared with PPI treatment. WMT significantly relieved the symptoms of NERD in patients, reduced PPI dependency, prolonged the duration of symptom remission, and reduced symptom recurrence.

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is divided into nonerosive reflux disease (NERD), reflux esophagitis, and Barrett's esophagus, and more than 70% of GERD cases are NERD[1]. The current treatment for GERD is proton pump inhibitors (PPIs)[2]. However, due to the chronic nature and recurrence of GERD, current PPI treatments for GERD do not provide satisfactory effects, especially in patients with NERD, who have a poorer response to PPIs than patients with reflux esophagitis[3-7]. In addition, many patients need to take PPIs for a long time[8], potentially leading to changes in their intestinal microbiota, such as increases in *Enterococcus*, *Streptococcus*, *Staphylococcus*, and potentially pathogenic *Escherichia coli*[6,9]. *Escherichia coli* is a Gram-negative bacterium, and a component of its cell wall, lipopolysaccharide, can cause lower esophageal sphincter (LES) relaxation and gastric empty-out delay and lead to the occurrence of GERD[6]. Many studies have also found that long-term use of PPIs can result in small intestinal bacterial overgrowth (SIBO)[9-11], which can cause chronic inflammation; immune reactions may give rise to reduced reactivity of esophageal smooth muscle[12]. At the same time, SIBO leads to the production of excess amounts of methane gas by the intestinal bacteria, which inhibits contractile activity, slows intestinal transit, and, consequently, affects gastric emptying and induces gastroesophageal reflux[13,14]. These studies suggest that the recurrence of symptoms and long-term use of PPI in patients with GERD (including NERD) may be related to changes in the intestinal microbiota and SIBO induction after previous PPI use.

In recent years, research on the application of fecal microbiota transplantation (FMT) in clinical diseases has developed rapidly. Good clinical effects of FMT have been observed for many diseases, including refractory *Clostridium difficile* infection[15], irritable bowel syndrome[16], inflammatory bowel disease[17], and constipation[18]. FMT reconstructs the balance of the intestinal flora in patients by using healthy donor feces,

reducing the colonization of pathogenic bacteria, and leading to a treatment effect by regulating metabolism and immunity[19]. The latest study showed that the method of generating bacterial solutions through automatic purification systems is very popular among doctors and patients; therefore, FMT is also called washed microbiota transplantation (WMT)[20].

Since our hospital launched WMT to treat diseases such as GERD, ulcerative colitis, irritable bowel syndrome, constipation, non-alcoholic fatty liver, and autism for two years and obtained good clinical results, more than 2500 times have been treated. This study mainly assessed the therapeutic effects of WMT in patients with PPI-dependent NERD.

MATERIALS AND METHODS

Subjects

Patients with a clear diagnosis of NERD and dependency on PPIs who were admitted to the Department of Gastroenterology of the First Affiliated Hospital of Guangdong Pharmaceutical University from January 1, 2017 to November 30, 2018 and had poor curative effects and recurrent symptoms after PPI use were included and divided into WMT and PPI groups. Patients who received WMT treatment provided informed consent.

Inclusion criteria

The age of the included patients was 18-85 years old. Consistent with the diagnosis of GERD, endoscopy excluded reflux esophagitis and Barrett's esophagus. The patients had a history of disease for more than 6 mo with PPI dependency, and no antibiotics were used 1 wk before treatment and during treatment. PPI dependency was defined that after standard PPI treatment, complete remission of GERD symptoms was not achieved or the symptoms recurred after the drug was stopped. The inclusion criteria of the WMT group were to meet both the above criteria and the following criteria: Patients had consented to WMT treatment, if they were not included in the PPI group.

Exclusion criteria

Patients were excluded if they had severe heart and lung disease, liver and kidney failure, malignant tumors, pregnancy, or other diseases that significantly affect quality of life, or they refused or failed to complete the follow-ups.

Treatment plan

The PPI group was treated with a previous treatment plan (mainly PPI treatment), while WMT treatment was added in the WMT group. The source of the bacterial suspension for WMT was mixed multidonor feces. All donors were healthy people aged 18 to 25 years, and they were required to undergo health examinations to exclude digestive tract diseases, tumors, infectious diseases, metabolic diseases, genetic diseases, and other related diseases and not to take antibiotics, as well as drugs that affect digestive tract dynamics and/or cause intestinal microecological disorders, for the last 3 mo. Two hundred milliliters of fresh fecal liquid was separated using an automatic purification system (GenFMTer; FMT Medical, Nanjing, China), and the prepared bacteria were injected into the patient's intestine *via* the middle or lower digestive tract within half an hour. There were two transplantation routes. One was the middle-gut route: Transendoscopic enteral tubing was placed in the jejunum under gastroscopy, and PPIs (such as lansoprazole 30 mg + normal saline 100 mL) were administered intravenously 1 h before injection of bacteria (to reduce the inactivation of bacteria when moving through the stomach). Metoclopramide hydrochloride (10 mg) was injected intramuscularly (to reduce adverse reactions such as vomiting or abdominal distension caused by irritation of the gastrointestinal tract by the bacterial fluid). The patients were placed in a sitting position when injecting the bacterial solution. The injection process was slow, requiring an injection time of at least 30 min for 200 mL of bacterial solution. After the injection, the patient was asked to remain sitting or standing for at least 2 h. The other route was the lower gut route: Transendoscopic enteral tubing was placed into the caecum *via* enteroscopy. When injecting the bacterial solution, the patient was in the right lateral position, and the time of injection was 30 min. After the injection was completed, the patient was instructed to rest in the right lateral position for at least 2 h. One course was administered once daily for 3 d. Four courses were administered, with one course per

month given in the first month, second month, third month, and sixth month.

Outcome measurement

The main outcome measure was NERD symptom frequency evaluated 1 mo after WMT. The reflux disease questionnaire (RDQ) and GERD questionnaire (GERDQ) were administered to the patient before and 1 mo after treatment and reviewed. The RDQ integrates the severity and frequency of heartburn, chest pain, acid regurgitation, and regurgitation over the past 4 wk. The GERDQ measures the frequency of symptoms, such as reflux, heartburn, nausea, upper abdominal pain, sleep disturbance, additional antacids, and other symptoms during the past 7 d.

Other outcome measures included remission, relapse after remission, monosymptomatic remission, and biochemical coupling examination of intestinal barrier function. Remission was defined as RDQ and GERDQ scores reduced by 30% at 1 mo posttreatment. Relapse after remission was defined as: After WMT treatment, the patient's symptoms of NERD reached remission but then worsened to a level observed prior to WMT treatment; the RDQ and GERDQ scores increased by 30% from the previous period, and the duration of this increase exceeded 1 mo. Monosymptomatic remission was defined when the RDQ or GERDQ scores of heartburn, acid regurgitation, chest pain, regurgitation, and sleep disturbance declined posttreatment compared with pretreatment. Biochemical coupling examination of intestinal barrier function was performed by determining serum levels of diamine oxidase (DAO), D-lactic acid (DLA), and lipopolysaccharide[21] according to the test developed by the Institute of Biophysics, Chinese Academy of Sciences (Beijing, China), and the manufacturer's protocol. DAO > 10 U/L indicated intestinal mucosal damage and increased intestinal permeability; DLA > 15 mg/L indicated abnormal intestinal permeability; and lipopolysaccharide > 20 U/L indicated intestinal bacterial translocation[22]. Thus, abnormal levels of any of these indicators reflect intestinal mechanical barrier dysfunction. PPI medication status, side effects of WMT treatment, and WMT course were also determined.

Statistical analysis

SPSS version 20.0 or GraphPad Prism version 5.0 was used for data analyses. The data are described as frequencies, percentages, medians, and interquartile ranges. Comparisons of lipopolysaccharide values and scores of RDQ and GERDQ were performed by applying the non-parametric Wilcoxon signed-rank test or Mann-Whitney test, and a two-tailed P value of < 0.05 was considered statistically significant.

Ethics statement

This retrospective study was approved by the Ethics Committee of Guangdong Pharmaceutical University (Approval No. Yilun Shen [2019] No. 93 01).

RESULTS

Of the 61 patients with NERD who were screened, 51 were eligible according to the inclusion criteria, and 27 completed the follow-ups (Figure 1). The WMT group included 15 patients, and the PPI group comprised 12 patients. There were no significant differences in the characteristics of the two study groups at enrolment (Table 1).

At 1 mo after treatment, the total remission rate in the WMT and PPI groups was 93.3% *vs* 41.7%. Compared with the PPI group, the WMT group showed better results in the GERDQ scores (7 *vs* 11, $P = 0.004$) and RDQ scores (8 *vs* 20.5, $P = 0.003$), as well as in the remission months [8 (3, 17) *vs* 2 (0, 4), $P = 0.002$] (Table 2); nine patients showed sustained remission for more than 6 mo in the WMT groups, while there were only two in the PPI group. Furthermore, the patients in the WMT group achieved better improvements in heartburn (9/10 *vs* 7/11), acid regurgitation (12/14 *vs* 7/11), chest pain (5/6 *vs* 1/5), regurgitation (9/12 *vs* 4/8), and sleep disturbance (9/11 *vs* 5/5) than the PPI group. However, 13.3% (2/15) of patients in the WMT group relapsed after remission (Table 3).

The PPI dose was reduced to some extent in 80% (12/15) of the patients in the WMT group and 33.3% (4/12) in the PPI group. After receiving the WMT treatment, 72.7% (8/11) of the patients who continued using PPIs reduced their PPI doses, and all four patients with on-demand PPI use were also reduced. In addition, 33.3% (5/15) of the patients maintained symptomatic relief and stopped taking PPIs in the WMT group, while the percentage was 16.7% (2/12) in the PPI group (Table 4).

Table 1 Basic information of patients with nonerosive reflux disease

Item	PPI	WMT	*P* value
Female	6	8	
Age (median; IQR, yr)	58.5 (53, 67.25)	62 (55, 67)	0.526
Duration (median; IQR, mo)	48 (15, 144)	24 (6, 60)	0.218
BMI (median; IQR, kg/m^2)	21.63 (19.13, 24.38)	24.24 (22.58, 25.43)	0.079

WMT: Washed microbiota transplantation; PPI: Proton pump inhibitors; BMI: Body mass index; IQR: Interquartile range.

Table 2 Symptom scores and remission time before and after treatment in the two groups

Group	PPI (*n* = 12)	WMT (*n* = 15)	*P* value[1]
Pre-GERDQ (median; IQR)	12 (9, 13.75)	12 (10, 12)	0.746
Post-GERDQ (median; IQR)	11 (8.25, 13.5)	7 (7, 8)	0.004
Intra-group, *P* value[2]	0.477	0.003	
Pre-RDQ (median, IQR)	26 (18, 33)	23 (16, 25)	0.203
Post-RDQ (median, IQR)	20.5 (12.75, 26)	8 (6, 12)	0.003
Intra-group, *P* value	0.005	0.002	
Remission time (median, IQR; mo)	2 (0, 4)	8 (3, 17)	0.002

[1]The RDQ and GERDQ scores between two groups were analyzed by non-parametric Mann-Whitney test.
[2]The intra-group RDQ and GERDQ scores were analyzed by non-parametric Wilcoxon signed-rank test.
WMT: Washed microbiota transplantation; PPI: Proton pump inhibitors; RDQ: Reflux disease questionnaire; GERDQ: Gastroesophageal reflux disease questionnaire; IQR: Interquartile range.

Table 3 Clinical responses to washed microbiota transplantation in patients with nonerosive reflux disease

Item		WMT, *n* = 15
Remission[1], *n*		14 (93.3)
Remission after the first course of WMT, *n*		13 (86.7)
Remission after the first course of FMT until the end of the study, *n*		10 (66.7)
No remission after the first course of WMT, *n*		2 (13.3)
Recurrence after remission, *n*		2 (13.3)
Side effects of WMT treatment[2], *n*	No	14 (93.3)
	Yes	1 (6.70)

[1]The scores of the reflux disease questionnaire or gastroesophageal reflux disease questionnaire at 1 mo post-washed microbiota transplantation (WMT) were reduced by 30%.
[2]Only one of the 15 patients had minor side effects, with a mushy stool two or three times a day, which resolved on their own after 1 wk, but no abdominal pain, black stool, fever, or serious WMT-related side effects were observed.
WMT: Washed microbiota transplantation; FMT: Fecal microbiota transplantation.

With regard to the relationship between courses of WMT and remission in patients with NERD symptoms (Figure 2), ten people who completed three or more WMT courses achieved an 80% symptom remission rate. With increasing courses of WMT treatment, the remission rate increased.

Twenty-four patients had the function of the intestinal mucosal barrier examined before treatment, which showed that over half of the patients (13/24) had different degrees of intestinal mucosal barrier function damage, such as the change of epithelial permeability (8/24), the damage of intestinal epithelial cells (4/24), intestinal mucosal

Table 4 Use of proton pump inhibitors in nonerosive reflux disease patients

Items		PPI	WMT
Continuous use before treatment (*n*)	No reduction after treatment	3	4
	Reduction after treatment	5	0
	Withdrawal after treatment	3	0
Usage as needed before treatment (*n*)	No reduction after treatment	0	4
	Reduction after treatment	2	2
	Withdrawal after treatment	2	2

WMT: Washed microbiota transplantation; PPI: Proton pump inhibitors.

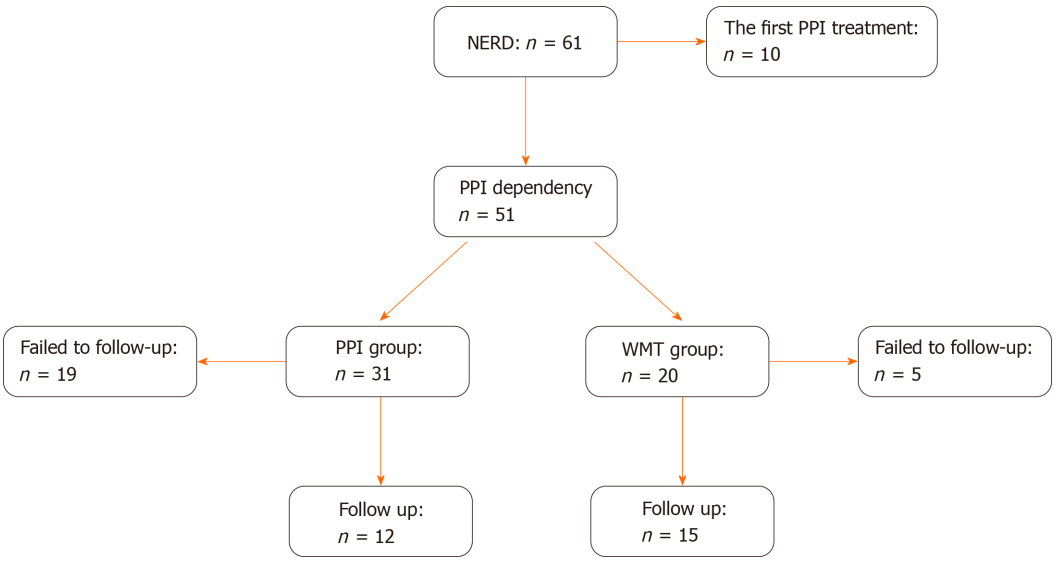

Figure 1 Flow chart for the inclusion of patients. NERD: Nonerosive reflux disease; WMT: Washed microbiota transplantation; PPI: Proton pump inhibitor dependency.

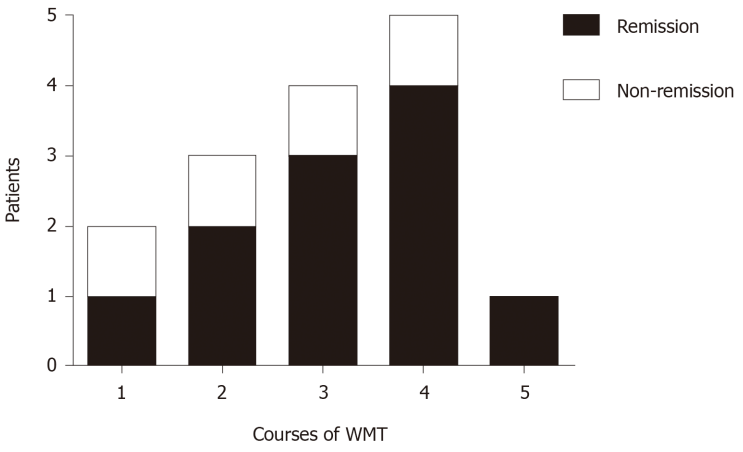

Figure 2 Relationship between courses of washed microbiota transplantation and remission of nonerosive reflux disease. WMT: Washed microbiota transplantation.

ischaemia (4/24), and intestinal bacterial translocation (2/24). However, in the WMT group, eight samples were analyzed, and the function of the intestinal mucosal barrier observed as being worse in the post-treatment than pre-treatment, two patients before treatment with intestinal mucosal damage were positive to negative, while six patients with intestinal mucosal barrier damage were negative to positive. Wilcoxon signed-rank test showed that the numeric values of lipopolysaccharide, diamine oxidase, and D-lactate had no significant difference between before and after WMT treatment (Table 5).

Only one of the 15 patients had minor side effects, including a mushy stool two or three times a day, which resolved on their own after 1 wk, but no abdominal pain, black stool, fever, or serious WMT-related side effects were observed.

DISCUSSION

PPIs are currently the first-line treatment for GERD. NERD, which accounts for most GERD cases, ruins patients' quality of life and is more difficult to treat than reflux esophagitis[23]. However, not all patients with NERD can achieve good treatment effects because PPIs are less effective in patients with NERD than in those with reflux esophagitis, and its chronic and recurrent nature means that some patients need to take PPIs for a long time. In addition, potential side effects of long-term use of PPIs are gradually being revealed, including SIBO, lack of micronutrients, and dementia[9,11,24]. A study with population-based follow-up for 10 years in Hong Kong, China in 2018 showed that long-term PPI use could increase the risk of gastric cancer[25]. Therefore, new challenges are arising for NERD treatment, and the results of our study show that WMT can solve this problem. This study confirmed that WMT had a significant clinical effect on NERD in patients with PPI dependency compared to PPI alone. It can reduce the PPI dose and the NERD recurrence rate. Our results showed that WMT, combined with PPI treatment, resulted in remission in 93.3% (14/15) of the patients, and 80% (12/15) of the patients reduced the PPI dose, which was far better than that of the PPI group (33.3%). Compared with the PPI group, the WMT group showed better results in the GERDQ scores and RDQ scores as well as in the remission months, which had obvious statistical significance.

It is widely believed that GERD usually occurs through the following four mechanisms[26]: Transient LES relaxation, low LES pressure, swallowing-associated LES relaxation, and straining during periods with low LES pressure. Recently, new findings related to the pathogenesis of GERD showed that lipopolysaccharide, a component of Gram-negative cell walls, is involved in the mechanism of GERD. Research has shown that the state of esophageal diseases (oesophagitis, Barrett's esophagus, *etc*.) is mainly dominated by type II Gram-negative bacteria. We know that lipopolysaccharide is the main structure of the outer membrane in Gram-negative bacteria; it can activate Toll-like receptor 4 and the downstream nuclear factor kB pathway to induce an inflammatory response and upregulate the expression of inducible nitrous oxide synthase, which relaxes the LES, and COX-2, which delays gastric emptying[27]. In this retrospective study, 24 patients had the function of the intestinal mucosal barrier examined before treatment, which showed that over half of the patients (13/24) had different degrees of intestinal mucosal barrier function damage, which may be involved in the pathogenesis of GERD. We also analyzed the values of lipopolysaccharide, diamine oxidase, and D-lactate before and after WMT treatment for 8 patients. The results showed that the medians were increased after treatment compared with those before treatment, but there was no significant difference. This seems to be contrary to evidence from a previous study. However, this may be due to the small number of samples, and the test was not performed at the same time. This result gives us good inspiration to focus on researching this aspect in the future.

The results of this trial indicate that as the number of courses of WMT treatment increases, the remission rate increases. The response rate of patients who completed more than four courses of WMT reached 83.3%, but the results may have some limitations due to the small sample size in this study. Although WMT is increasingly used in clinical treatment, there is no uniform treatment course or dosage. According to previous reports, multiple fresh fecal transplants can improve clinical efficacy[28]. We recommend that WMT for GERD be administered in four courses at 1, 2, 3, and 6 mo (1 course is a continuous fecal treatment for 3 d, with 200 mL of fecal separated solution injected into the jejunum or caecum *via* the middle or lower digestive tract per day). The four courses of treatment recommended in this study are feasible, and it seems

Table 5 Results of examining the function of the intestinal mucosal barrier in the washed microbiota transplantation group

Item	Lipopolysaccharide	Diamine oxidase	D-lactate
Pre-treatment (median, IQR)	7.5 (3.6, 10.4)	4.1 (3.2, 6.5)	8.9 (7.6, 11.8)
Post-treatment (median, IQR)	10.1 (1.0, 23.6)	7.7 (2.3, 13.54)	16.3 (7.7, 18.3)
P value[1]	0.401	0.263	0.208

[1]The results were analyzed by nonparametric Wilcoxon signed-rank test.
IQR: Interquartile range.

that increasing the treatment course can increase the rate of remission. The poor WMT responses observed in this study may be related to the existence of organic lesions that cause GERD, such as hiatal hernia, in some patients, and some may be related to failure to complete the recommended courses.

Because this is a retrospective study, its limitations are that the accuracy and completion of the relevant data and questionnaires could not be improved during follow-up. Memory bias may have affected the questionnaires. The analysis of related test items had a small sample size, which affects the analysis of the experimental results.

CONCLUSION

In conclusion, this study for the first time demonstrated that WMT is safe and effective in treating patients with NERD and PPI dependency. Compared to the PPI group, it can significantly relieve the symptoms of NERD patients, reduce PPI dependency, prolong the duration of remission in symptoms, and reduce recurrence. It can also increase the diversity and evenness of the bacterial community. The next step is to expand the sample size and perform further studies to confirm the role of WMT in the treatment of NERD and elucidate its mechanism.

ARTICLE HIGHLIGHTS

Research background

The pathogenesis of gastroesophageal reflux disease (GERD) is closely associated with the intestinal bacteria composition and their metabolites.

Research motivation

At present, the treatment of GERD has not achieved satisfactory clinical results.

Research objectives

To investigate whether washed microbiota transplantation (WMT) improves symptoms of nonerosive reflux disease (NERD) with proton pump inhibitor (PPI) dependency.

Research methods

Patients with recurrent NERD and PPI dependency at the First Affiliated Hospital of Guangdong Pharmaceutical University from 2017 to 2018 were included and divided into a WMT or PPI group treated with PPI with/without WMT. The endpoint was NERD symptom frequency evaluated 1 mo after WMT using reflux disease questionnaire (RDQ) and GERD questionnaire (GERDQ) scores, remission time, PPI dose, and the examination of intestinal mucosal barrier function.

Research results

In the WMT (n = 15) and PPI (n = 12) groups, the total remission rate at 1 mo after treatment was 93.3% *vs* 41.7%. Compared with the PPI group, the WMT group showed better results in GERDQ (P = 0.004) and RDQ (P = 0.003) and in remission months (8 *vs* 2, P = 0.002). The PPI dose was reduced to some extent for 80% of patients in the WMT group and 33.3% in the PPI group. In 24 patients, intestinal mucosal barrier function

was examined before treatment, and changes in the degree of damage were observed in 13 of these patients after treatment. Only one of the 15 patients had minor side effects, including a mushy stool two or three times a day, which resolved on their own after 1 wk.

Research conclusions

This study is the first to demonstrate that WMT may be safe and effective for relieving NERD symptoms and reducing PPI dependency and recurrence.

Research perspectives

WMT could be a new treatment for NERD.

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ORIGINAL ARTICLE

Prospective Study

Prevalence of advanced liver fibrosis and steatosis in type-2 diabetics with normal transaminases: A prospective cohort study

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Author contributions: Makker J and Tariq H designed and performed the study, analyzed the data, and drafted the manuscript; Kumar K and Ravi M collected the clinical data and performed Liver elastography; Hayat U and Patel H performed the bio statistical analysis; Shaikh DH, Hassan MT, Patel H and Leung V made critical revisions to the manuscript; Nayudu S and Chilimuri S supervised the study performance, reviewed the manuscript and gave final approval of the version of the article to be published.

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Abstract

BACKGROUND

Nonalcoholic fatty liver disease (NAFLD) and type-2 diabetes mellitus (T2DM) have an intricate bidirectional relationship. Individuals with T2DM, not only have a higher prevalence of non-alcoholic steatosis, but also carry a higher risk of progression to nonalcoholic steatohepatitis. Experts still differ in their recommendations of screening for NAFLD among patients with T2DM.

AIM

To study the prevalence of NAFLD and advanced fibrosis among our patient population with T2DM.

METHODS

During the study period (November 2018 to January 2020), 59 adult patients with T2DM and 26 non-diabetic control group individuals were recruited prospectively. Patients with known significant liver disease and alcohol use were excluded. Demographic data and lab parameters were recorded. Liver elastography was performed in all patients.

RESULTS

In the study group comprised of patients with T2DM and normal alanine aminotransferase levels (mean 17.8 ± 7 U/L), 81% had hepatic steatosis as diagnosed by elastography. Advanced hepatic fibrosis (stage F3 or F4) was

04).

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present in 12% of patients with T2DM as compared to none in the control group. Patients with T2DM also had higher number of individuals with grade 3 steatosis [45.8% *vs* 11.5%, (P < 0.00001) and metabolic syndrome (84.7% *vs* 11.5%, P < 0.00001)].

CONCLUSION

A significant number of patients with T2DM, despite having normal transaminase levels, have NAFLD, grade 3 steatosis and advanced hepatic fibrosis as measured by liver elastography.

Key Words: Advanced liver fibrosis; Diabetes; Steatosis; Normal transaminases; Fatty liver disease; Metabolic syndrome

Core Tip: Individuals with type 2 diabetes mellitus (T2DM) have a higher prevalence of non-alcoholic steatosis and a higher risk of progression to non-alcoholic steatohepatitis and cirrhosis. Experts differ in their screening recommendations for nonalcoholic fatty liver disease among patients with T2DM. We prospectively recruited and performed liver elastography on 59 diabetics and 26 non-diabetic control patients. Patients with known liver disease and alcohol use were excluded. Our study shows advanced fibrosis is prevalent among patients with T2DM as compared to non-diabetics, even with normal liver enzymes. Screening for liver fibrosis in all patients with T2DM should be considered, regardless of liver enzyme levels.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of clinico-pathological conditions ranging from simple fatty liver disease to nonalcoholic steatohepatitis (NASH). The risk of progression of NASH to cirrhosis has previously been estimated at 21%-26% over a mean follow up of 8.2 years[1]. With the globalization of western diets and increasingly sedentary lifestyles, obesity rates have skyrocketed to epidemic proportions. In parallel with obesity, the prevalence of NAFLD has dramatically increased. The prevalence of NAFLD worldwide ranges from 6% to 35% depending on the method used for diagnosis and the study population[2]. The prevalence in United States has ranged between 11%-34% with the most recent study reporting a prevalence of 46%[3]. As a result of its increasing prevalence, NAFLD has now become the leading cause of liver disease in the western countries[2]. With the rising rates of NAFLD, and the availability of effective durable treatment options for other chronic liver diseases like chronic hepatitis C, experts have predicted NAFLD to become the leading cause of liver transplantation in the near future.

NAFLD and type-2 diabetes mellitus (T2DM) are intricately related to each other and this relationship is bidirectional. Development of insulin resistance is a key phenomenon underlying NAFLD as well as T2DM and as a result, these two disorders commonly occur together. Individuals with T2DM have a higher prevalence of NAFLD[4]. Individuals with T2DM who develop non-alcoholic steatosis also carry a higher risk of progression to NASH[5]. Development of NASH is characterized by progressive hepatocyte necroinflammation which is an important harbinger of cirrhosis and its associated complications such as hepatocellular cancer. In a study from the United States involving 1249 patients with T2DM and biopsy proven NAFLD, prevalence of NASH and advanced liver fibrosis was 69.2% and 41% respectively[6]. A Canadian study with a 12-year follow-up involving nearly 2.5 million

participants, among which nearly one-fifth had newly diagnosed T2DM, found more than two-fold risk of incident liver cirrhosis in patients with newly diagnosed T2DM[7].

Despite the alarming rise in NAFLD prevalence among patients with T2DM, major societies differ in their recommendations about screening for NAFLD among patients with T2DM. The American Association for the Study of Liver Diseases does not recommend NAFLD screening in individuals with T2DM due to its unknown long-term benefits and cost-effectiveness[8]. On the other hand, American Diabetes Association recommends assessment of liver fibrosis in patients with T2DM and elevated transaminases[9]. Our study was conducted to explore the prevalence of NAFLD and advanced fibrosis among our minority inner city population with T2DM who had normal transaminases.

MATERIALS AND METHODS

Study group

From November 2018 to January 2020, all patients aged 18 years and above with T2DM attending the endocrine clinic at our hospital were offered enrollment in this prospective cohort study. All patients who agreed, provided a written and informed consent for their participation. Patients with a known diagnosis of cirrhosis, chronic hepatitis B, chronic hepatitis C, autoimmune hepatitis, drug induced hepatitis, cholestatic liver disease, hemochromatosis and alcoholic liver disease were excluded. Patients with significant alcohol use defined as more than 20 g of daily alcohol use were excluded. Furthermore, any patient with alanine transaminase (ALT) level more than 40 (upper limit of normal at our lab) was excluded.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in the Bronx Care Health System Institutional Review Board's approval (IRB #05 10 18 04).

Control group

The control group comprised of patients without T2DM and normal ALT level (< 40 U/L) as defined by our lab. Patients were recruited from the gastroenterology clinic at our hospital. These patients had no known history of fatty liver disease. Exclusion criteria similar to the study group were applied.

Clinical and laboratory parameters

Demographic data including age, gender and ethnicity were collected. All comorbid medical conditions and a complete drug history were recorded *via* patient interviews. A physical examination including measurement of waist circumference, weight, height and body mass index (BMI) was performed at the time of inclusion. Laboratory data including liver enzymes, hemoglobin A1c, lipid profile available within one year of enrollment were recorded.

Assessment of hepatic steatosis and fibrosis using transient elastography

All patients were required to fast for three hours prior to elastography. Liver elastography was performed using the M or XL probe from Fibroscan 502 touch model (Echosens). The probe is automatically selected by the software based on thoracic perimeter and skin capsule distance measurements. Liver stiffness measurement and controlled attenuation parameter (CAP) which estimates the amount of liver fat was obtained. Elastography examination was considered reliable if at least 10 measurements were taken with an interquartile range interval per median LS less than 30%. Liver stiffness and CAP are calculated as a median over minimum 10 validated measurements and expressed in kilopascals (Kpa) and decibel per meter (db/m), respectively. Cut off for stage F0-1, F2, F3 and F4 fibrosis were ≤ 7 Kpa, ≥ 7.5 Kpa, ≥ 10 Kpa and ≥ 14.0 Kpa, respectively[10]. Cut off for CAP to diagnose hepatic steatosis grade 1 (5%-33% steatosis), grade 2 (34%-66% steatosis) and grade 3 (> 66% steatosis) were 238 db/m, 259 db/m and 290 db/m respectively[11].

Metabolic syndrome

Metabolic syndrome was defined by the National Cholesterol Education Program's Adult Treatment Panel III criteria. It is defined as presence of at least three of the following criteria: Waist circumference > 102 cm in men or > 88 cm in women, plasma triglycerides ≥ 150 mg/dL, high density lipoprotein cholesterol < 40 mg/dL in men or < 50 mg/dL in women, blood pressure ≥ 130/85 mmHg, and fasting plasma glucose ≥

110 mg/dL[12].

Sample size and statistical analysis

Sample size estimation was based on the assumption that prevalence of advanced fibrosis among patients with T2DM is around 20%. For a power of 80% at 5% significance level, 57 patients were required in the study group.

Statistical analysis was performed using statistical analysis system software, version 9.4 and graphpad prism software version 8.4.3. Frequencies and percentages were reported for categorical variables. Mean and standard deviations were reported for numerical continuous variables. Dichotomous variables were compared by chi-square analysis using the pearson test. A two-tailed value of < 0.05 was considered statistically significant. Bivariate analysis was done using analysis of variance to determine predictors of advanced fibrosis.

RESULTS

The study group comprised of 59 patients, whereas the control group had 26 patients. Demographics and baseline characteristics of both the groups were compared as shown in Table 1. The mean duration of T2DM in the study group was 15 ± 9 years. Diabetic microvascular complications including retinopathy, nephropathy, and neuropathy were prevalent in 22%, 30% and 17% of study patients, respectively. 41% of these patients were on insulin, 59% on metformin, 17% on sulfonylureas, 20% on glucagon-like peptide 1 receptor agonists, 8% on pioglitazone, 12% on sodium-glucose cotransporter-2 inhibitors, and 19% on dipeptidyl peptidase 4 inhibitors. Mean BMI of patients in the study group was significantly higher as compared to those in the control group (33.1 ± 8.4 *vs* 27.6 ± 4.7, $P = 0.0002$). A total of 7 patients in the study group were not obese and had BMI < 25, 18 patients were with BMI: 25-29.9, 11 had a BMI: 30-34.9, 14 had a BMI: 35-39.9, and 9 patients had a BMI of > 40.

Prevalence of hepatic fibrosis

Elastography revealed that 76% of our study group patients had absent or low-grade (F0-1) fibrosis, 12% had F2 stage fibrosis, 5% had F3 stage fibrosis, and 7% had F4 stage fibrosis. Advanced fibrosis stage (F3 or F4) was diagnosed in a total of seven (12%) patients from the study group (Figure 1). In the control group none of the patients had advanced stage fibrosis, 2 (8%) patients had F2 fibrosis and 24 (92%) patients had F0-1 stage fibrosis (Figure 2).

Prevalence of hepatic steatosis

Eighty-one percent of our study group patients with T2DM had hepatic steatosis as diagnosed by liver elastography. Steatosis grade 3 was prevalent in 27 (46%) patients as compared to 3 (12%) patients in the control group (P value < 0.00001). Steatosis stage 0, 1, and 2 were diagnosed in 11 (19%), 8 (13%), and 13 (22%) patients respectively in the study group (Figure 3). In comparison, the control group had 11 (42%), 7 (27%), and 5 (19%) patients with steatosis stage 0, 1, and 2 respectively.

Prevalence of metabolic syndrome

Metabolic syndrome was more prevalent among the study group patients. Fifty (85%) patients were diagnosed with metabolic syndrome in the study group and on the contrary only three (12%) patients in the control group had metabolic syndrome (P < 0.00001). Among the 27 (46%) patients in the study group who had S3 steatosis, 26 (96%) had metabolic syndrome. Twenty-eight out of the 59 (48%) patients who had metabolic syndrome in the study group met three criteria for metabolic syndrome, 13 (22%) patients met four criteria and remaining 9 (15%) patients met all the five criteria for metabolic syndrome (Figure 4).

Bivariate analysis was performed using analysis of variance to determine predictors of advanced fibrosis. Duration of T2DM was found to be a significant predictor (P < 0.05) of advanced fibrosis. Age ($P = 0.29$), BMI ($P = 0.30$), and waist circumference ($P = 0.46$) had no association with advanced fibrosis.

DISCUSSION

With the prevalence rate of obesity and T2DM reaching epidemic proportions, NAFLD

Table 1 Comparison of characteristics between control and study group

Variate	Control group, *n* = 26	Study group, *n* = 59	*P* value
Age (yr)	53 ± 12.3	62 ± 11.7	0.0014
Gender, *n* (%)			0.8099
Male	9 (35)	23 (39)	
Female	17 (65)	36 (61)	
Ethnicity, *n* (%)			0.5005
Hispanic	21 (81)	41 (69.5)	
African American	3 (12)	13 (22)	
Others	2 (7)	5 (8.5)	
Waist circumference (cm)	97.5 ± 8.4	98. 6 ± 18.5	0.7595
BMI (kg/m^2)	27.6 ± 4.7	33.1 ± 8.4	0.0002
Hypertension, *n* (%)	15 (58)	43 (73)	0.2083
Hyperlipidemia, *n* (%)	10 (38)	35 (59)	0.0999
ALT (U/L)	17 ± 7	17.8 ± 7	0.7189
Platelet count (10^9/L)	258 ± 81	237 ± 61	0.2007
Albumin (g/L)	4.4 ± 0.3	4.3 ± 0.3	0.0687
HDL (mg/dL)	57.7 ± 18.2	49.7 ± 15.5	0.0588
LDL (mg/dL)	106.3 ± 33.6	87.4 ± 35.5	0.0231
TG (mg/dL)	122.5 ± 79.3	154.7 ± 77.9	0.0883
HbA1c	5.4 ± 0.3	7.9 ± 1.8	< 0.0001
Liver stiffness using elastography (kPa)	5.2 ± 1.6	7.5 ± 9.6	0.2138
CAP (dB/m)	230 ± 70	284 ± 64	0.0008

BMI: Body mass index; ALT: Alanine transaminase; HbA1c: Hemoglobin A1c; CAP: Controlled attenuation parameter.

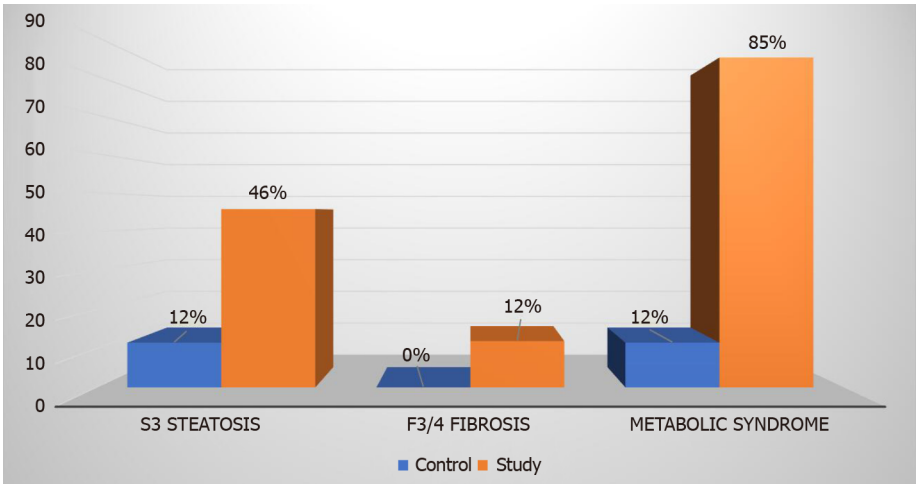

Figure 1 Comparison of S3 steatosis, F3/4 fibrosis and metabolic syndrome among control (non-diabetic) and study (diabetic) group.

has become a health crisis. NAFLD is seen frequently in T2DM with prevalence reported as high as 74% reported in one of the studies[13]. The pathophysiology of NAFLD and T2DM are intricately intertwined with insulin resistance serving as the common mediator. Severe hepatic necro-inflammation can progress to cirrhosis, especially more so in patients with T2DM. A study conducted by McPherson[14] has previously shown an alarmingly high rate of fibrosis progression not only in patients

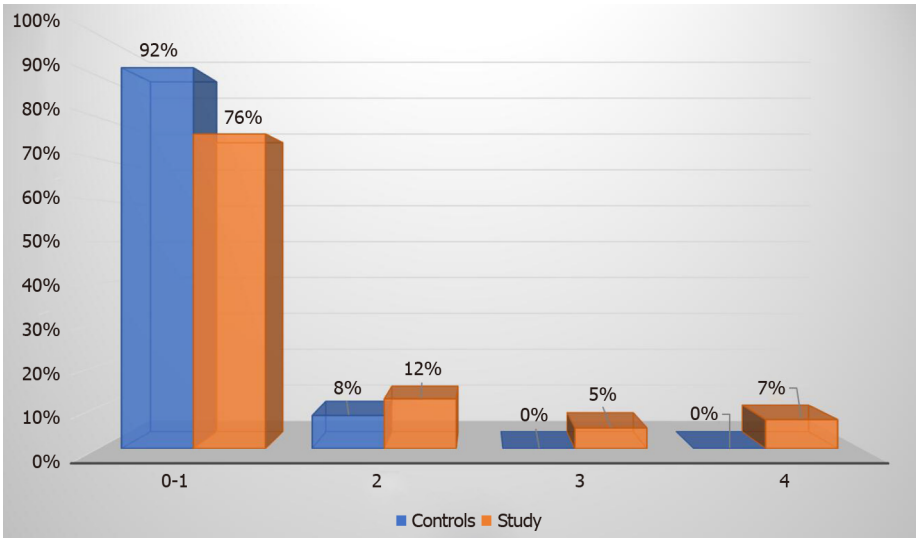

Figure 2 Control and study group patients stratified according to fibrosis stage.

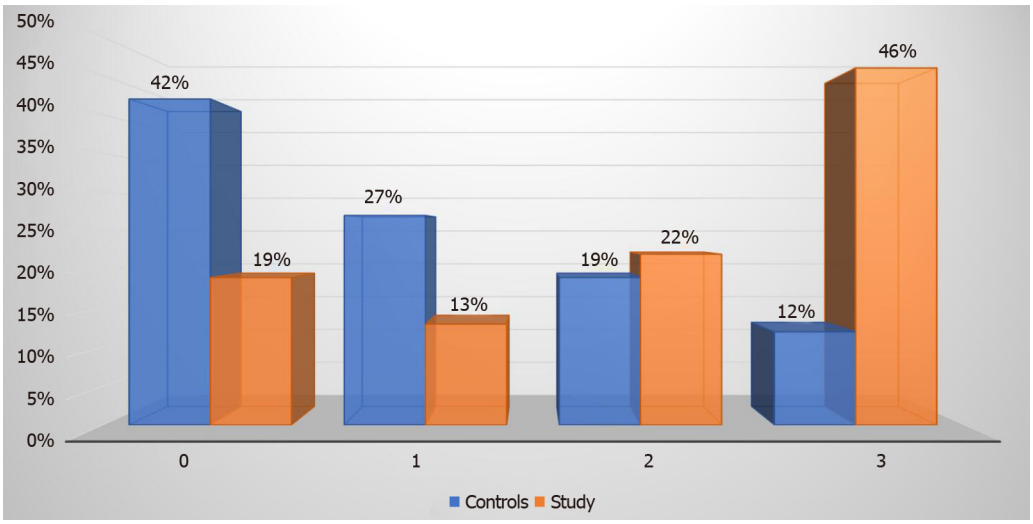

Figure 3 Control and study group patients stratified according to steatosis stage.

with NASH but also in individuals with NAFLD. The same study also showed that 22% of their patients who had NAFLD at their index liver biopsy showed progression to advanced fibrosis on their follow-up biopsy. Co-occurrence of NAFLD and T2DM poses a heightened risk of extrahepatic morbidity and mortality from cardiovascular disease, chronic kidney disease and hepatocellular cancer[15]. Despite these findings, guidelines from the expert societies differ in their opinion regarding the utility of screening for hepatic fibrosis in patients with NAFLD.

The prevalence of NAFLD in our study patients was higher than in previous reports, ranging from 40% to 70% in patients with T2DM[16]. Eighty-one percent of our patients with T2DM were found to have hepatic steatosis and nearly half of them had S3 steatosis. The prevalence of NAFLD in patients with T2DM previously reported in literature varies depending on the ethnicity and method used to diagnose NAFLD. Hispanics have been reported to have highest prevalence of non-alcoholic fatty liver disease[13] and more than 80% of our study patients were of Hispanic ethnicity.

Our study demonstrated that even patients with T2DM who had normal ALT levels were at risk of severe progressive liver disease. Twelve percent of our patients with T2DM had undiagnosed advanced fibrosis. Importantly, these patients who were noted to have advanced fibrosis had normal transaminases (mean ALT = 17.8 ± 7 U/L). Thus, based on current guidelines, these patients without clinically obvious cirrhosis would have gone undiagnosed for years. Among the seven study patients with BMI less than 25, five patients met criteria for metabolic syndrome, four patients

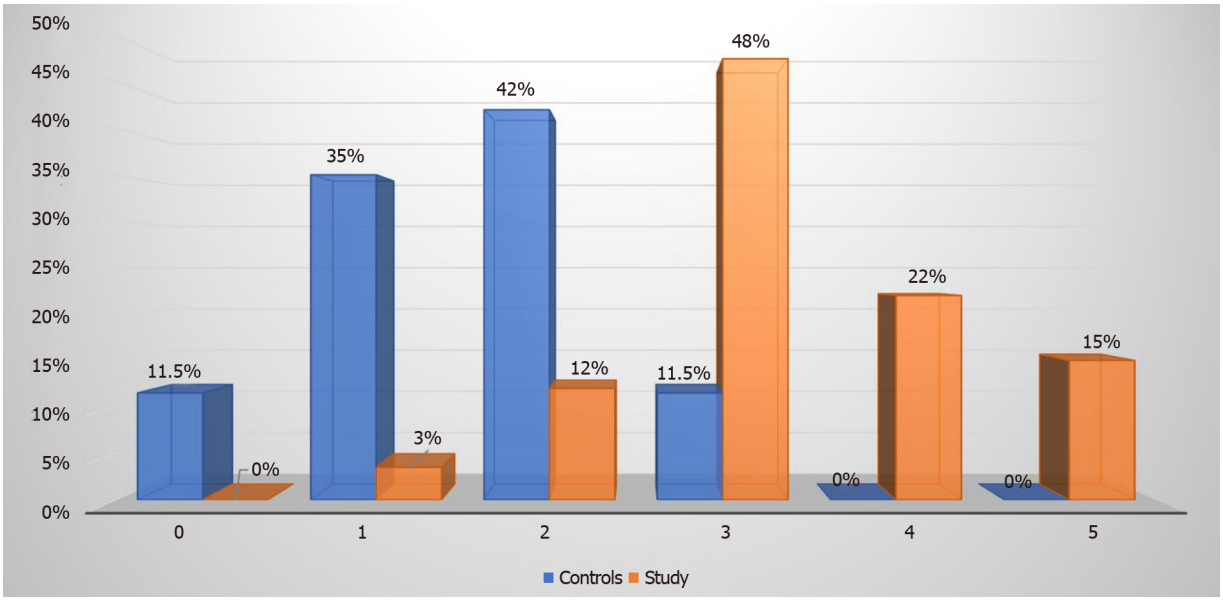

Figure 4 Control and study group patients stratified according to number of criteria met for metabolic syndrome.

had hepatic steatosis and one patient had advanced stage hepatic fibrosis. Thus, even non-obese patients with T2DM and normal transaminases could not be assumed to have a safer metabolic profile.

A cross sectional study done by Tuong *et al*[17] similarly studied the prevalence of hepatic fibrosis among patients with T2DM and found 73.3% of their diabetic patients to have NAFLD. The prevalence of F3 and F4 in their study group was 5.9% and 3.6% respectively, but they included patients with elevated transaminases as well, with a mean ALT of 60.6 U/L in the advanced fibrosis group. Another cross-sectional study by Lai *et al*[18] using transient elastography in patients with T2DM, showed a prevalence of cirrhosis and advanced fibrosis in 13.5% and 21% of patients, respectively. This study also included patients with elevated transaminases with a mean ALT of 38 U/L in patients with cirrhosis. A Turkish study found prevalence of advanced fibrosis of 16.9% and cirrhosis of 8% in their patients with T2DM. The mean ALT in their patients with F3 fibrosis was 39 U/L[19]. In a large prospective cohort study from Hong Kong involving 1918 patients with T2DM, increased liver stiffness suggestive of F3 or F4 fibrosis was found in 18.8% of their diabetic patients with normal ALT, defined as ALT less than 30 U/L in men and less than 19 U/L in women. This data is predominantly from an Asian population and does not reflect our study population[20]. In a study from Australia, prevalence of significant fibrosis, defined by the study as stage F2 and above, was 35% in diabetics. The mean ALT level was 38 U/L among these patients with significant fibrosis[21]. In a prospective French study, significant fibrosis defined as liver stiffness > 8.7 kPa was found in 12.9% of patients with T2DM. However, this study did not exclude patients with significant alcohol use and some of their patients found to have cirrhosis reported > 70 drinks of alcohol per week[22]. Prevalence of F4 stage fibrosis defined as liver stiffness > 9.5 kPA in another study from Romania was 18.6%. However, like most of the other studies, they too included patients with elevated ALT, with a significant number of patients having an ALT more than 40 U/L[23]. Currently, it is not the standard of care to assess liver fibrosis using liver biopsy in patients with normal ALT levels. However, it has been shown before that ALT is not a reliable criterion to exclude patients for assessment of liver fibrosis[24]. In this Italian study involving 458 liver biopsy confirmed NAFLD patients, 63 patients had normal ALT. NASH was demonstrated in 27% of those who had ALT levels less than 30 U/L in men and 19 U/L in women.

Liver biopsy is considered a gold standard diagnostic method for evaluating chronic liver diseases. In patients with NAFLD, it is particularly useful to assess for the presence of steatohepatitis which may progress to the development of cirrhosis. However, liver biopsy is an imperfect tool that is costly and invasive. It is associated with complications including pain in up to 50% of patients, serious bleeding in 0.6%, injury to other internal organs in about 0.08% and rarely death in 0.1% of patients[25]. Considering the enormous burden of NAFLD in our communities and the invasive nature of liver biopsy, it is not practically feasible to subject all patients with NAFLD

to liver biopsy. Surrogate non-invasive methods of liver fibrosis assessment have been developed and are widely available. Vibration controlled liver elastography is one such tool, that is commonly available in United States. It is a quick office-based procedure with acceptable intra-observer and inter-observer variability which has been validated worldwide[26].

We noticed striking gender differences in our study population. In general, higher prevalence of NAFLD has been reported in males as compared to female patients. However, when age specific gender differences are explored further, post-menopausal women have a higher prevalence of NAFLD as compared to men[27]. Our study results are consistent with these findings, demonstrating that 86% of all female patients as compared to 78% of all male patients had NAFLD. Among the 23 male patients in the study group (mean age of 63 years) only one (4%) patient had advanced fibrosis. In comparison, 36 patients were females (mean age of 62 years) and a total of six (17%) had advanced fibrosis. Female patients not only had higher prevalence of NAFLD, but also had a higher prevalence of grade 3 steatosis which was observed in 55% of females as compared to 30% of males. Although gender has been consistently shown to be a significant modifier, gender specific personalized therapy for NAFLD has not been explored and further research is needed to investigate this area.

Clinical implication

Considering the high prevalence of T2DM and NAFLD in our modern world, the burden of undiagnosed cirrhosis is enormous. Diagnosing these patients in a timely fashion would provide the opportunity to adopt intensive lifestyle modifications, and consequently avoid or at the least delay the progression to advanced hepatic fibrosis. Thus, it is important to recognize these patients early on, emphasize the progression of untreated NAFLD to liver cirrhosis, and potentially reduce morbidity, mortality and its related health care burden.

Study limitations

Our study has several limitations. Firstly, the number of overall patients in our study group is small and hence further studies with larger sample sizes are needed to validate our results. Second, we utilized liver elastography to diagnose NAFLD and liver biopsy was not performed in our patients, hence, we do not know the prevalence of NASH, which is the main driver for cirrhosis. However, it is not only unethical but risky too, to perform a liver biopsy in all T2DM patients especially with normal transaminase levels. Thirdly, the inclusion criteria in our study required a normal ALT level, defined as less than 40 U/L by our lab, which is higher than the current accepted standard. Nevertheless, the mean ALT level in the control and study group was 17 and 17.8 U/L, respectively. Fourthly, patients in our study group are older than the control group. Multiple studies have explored the influence of age on liver stiffness, and the results have been conflicting so far, reporting no difference across age groups[28,29], higher liver stiffness measurement in younger[30] or older[31] age. Lastly, our study and control group populations comprised predominantly of Hispanics and African Americans and hence these results cannot be generalized.

CONCLUSION

In conclusion, our study data shows that significant liver disease is prevalent among patients with T2DM, even with normal ALT levels. Measuring ALT levels in our study as a predictor of significant liver disease was unreliable. Timely work up with non-invasive techniques for estimation of liver fibrosis is warranted to prevent presentation at advanced stages of fibrosis. Primary care physicians and endocrinologists should be aware of this complication and should consider screening for liver fibrosis in all patients with T2DM, regardless of liver enzyme levels. Larger studies involving other ethnic groups are needed to validate our results.

ARTICLE HIGHLIGHTS

Research background

With the current obesity epidemic, prevalence of non-alcoholic fatty liver disease (NAFLD) has increased. Individuals with type 2 diabetes mellitus (T2DM) have a higher prevalence of non-alcoholic steatosis, may carry a higher risk of progression to

nonalcoholic steatohepatitis and eventually cirrhosis.

Research motivation

Experts still differ in their recommendations of screening for NAFLD among patients with T2DM.

Research objectives

To study the prevalence of NAFLD and advanced fibrosis among our patient population with T2DM with normal transaminases and without known liver disease.

Research methods

Prospective cohort study assessing hepatic steatosis and fibrosis using transient elastography in 59 patients with T2DM, compared to 26 non-diabetic control group patients.

Research results

In our study group, 81% of patients had hepatic steatosis and 12% had advanced fibrosis on liver elastography. In the control group none of the patients had advanced stage fibrosis. Grade 3 steatosis was prevalent in 46% of patients in the study group as compared to 12% patients in the control group (P value < 0.00001).

Research conclusions

A significant number of patients with T2DM, despite having normal transaminase levels, have advanced fibrosis or steatosis as measured by liver elastography.

Research perspectives

Physicians should be aware of prevalence of significant liver disease among patients with T2DM, even with normal liver enzymes and should consider earlier screening to prevent presentation at advanced stages of fibrosis. Larger studies are needed to confirm and validate our results.

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CASE REPORT

Pancreaticoduodenectomy after neoadjuvant chemotherapy for gastric cancer invading the pancreatic head: A case report

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Author contributions: Yura M and Takano K performed the surgery and wrote the paper; Koyanagi K and Nakagawa M reviewed the manuscript; Dehari R contributed to pathological diagnosis; All other authors equally contributed to medical treatment; All authors were responsible for the revision of the manuscript and final approval for submission.

Informed consent statement: Written informed consent was obtained from the patient for publication of this case report and any accompanying images.

Conflict-of-interest statement: All

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Abstract

BACKGROUND

Pancreaticoduodenectomy (PD) for advanced gastric cancer is rarely performed because of the high morbidity and mortality rates and low survival rate. However, neoadjuvant chemotherapy for advanced gastric cancer has improved, and chemotherapy combined with trastuzumab may have a preoperative tumor-reducing effect, especially for human epidermal growth factor receptor 2 (HER2)-positive cases.

CASE SUMMARY

We report a case of successful radical resection with PD after neoadjuvant S-1 plus oxaliplatin (SOX) and trastuzumab in a patient (66-year-old male) with advanced gastric cancer invading the pancreatic head. Initial esophagogastroduodenoscopy detected a type 3 advanced lesion located on the lower part of the stomach obstructing the pyloric ring. Computed tomography detected lymph node metastasis and tumor invasion to the pancreatic head without distant metastasis. Pathological findings revealed adenocarcinoma and HER2 positivity (immunohistochemical score of 3 +). We performed staging laparoscopy and confirmed no liver metastasis, no dissemination, negative lavage cytological findings, and immobility of the distal side of the stomach due to invasion to the pancreas. Laparoscopic gastrojejunostomy was performed at that time. One

authors declare no conflicts of interest.

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course of SOX and three courses of SOX plus trastuzumab were administered. Preoperative computed tomography showed partial response; therefore, PD was performed after neoadjuvant chemotherapy, and pathological radical resection was achieved.

CONCLUSION

We suggest that radical resection with PD after neoadjuvant chemotherapy plus trastuzumab is an option for locally advanced HER2-positive gastric cancer invading the pancreatic head in the absence of non-curative factors.

Key Words: Pancreaticoduodenectomy; Gastric cancer; Neoadjuvant chemotherapy; Trastuzumab; Human epidermal growth factor receptor 2; Case report

Core Tip: Because of the high surgical risk and poor prognosis, pancreaticoduodenectomy (PD) is rarely performed for gastric cancer. However, due to advances in surgery and improvements in perioperative management, PD may be considered for gastric cancer to improve long-term survival. We present the successful case of radical resection with PD after neoadjuvant chemotherapy combined with trastuzumab for human epidermal growth factor receptor 2-positive locally advanced gastric cancer invading the pancreatic head without postoperative severe complication. This case suggests that radical resection with PD after neoadjuvant chemotherapy combined with trastuzumab is an option for locally advanced human epidermal growth factor receptor 2-positive gastric cancer invading the pancreatic head in the absence of non-curative factors.

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INTRODUCTION

Gastric cancer is the fifth most frequent cancer and the third most frequent cause of cancer death worldwide, according to the global cancer statistics presented in 2018[1]. Curative resection of gastric cancer is essential to achieve long-term survival. In patients with locally advanced gastric cancer invading adjacent organs, extended multivisceral resection is required to achieve an R0 resection. Pancreaticoduodenectomy (PD) is theoretically needed to achieve an R0 resection for locally advanced gastric cancer with invasion to the head of the pancreas and duodenum. This procedure has rarely been performed for gastric cancer because of the significant morbidity and mortality and poor prognosis[2,3]; however, some recent studies have shown that resection combined with PD is associated with improved survival in patients with locally advanced gastric cancer[4-7]. Li *et al*[8] described in their recent systematic review that PD is a feasible option for locally advanced gastric cancer invading the duodenum and/or pancreas, with acceptable surgical risk, and offers survival benefits for selected patients. In addition, several randomized controlled studies showed that postoperative adjuvant chemotherapy for gastric cancer has improved the prognosis of advanced cases[9-11], and preoperative chemotherapy is also expected to be effective, with several clinical trials currently underway[12,13]. Therefore, multidisciplinary treatment is considered important especially for advanced cases, and the indications for PD to achieve an R0 resection for locally advanced gastric cancer invading the pancreas should be reconsidered.

However, there are no reports showing the therapeutic effect of PD on advanced gastric cancer after neoadjuvant chemotherapy especially in combination with trastuzumab. Our case is the successful report of radical resection with PD after

neoadjuvant S-1 plus oxaliplatin (SOX) combined with trastuzumab in a patient with locally advanced gastric cancer invading the pancreatic head.

CASE PRESENTATION

Chief complaints

A 66-year-old man had symptoms of abdominal pain, distension, and weight loss (from 62 to 47 kg within 6 mo).

History of present illness

The patient complained of abdominal distension and weight loss and had visited the hospital previously. Esophagogastroduodenoscopy (EGD) was performed, and an advanced type 3 lesion was detected on the lower part of the gastric body with stenosis, causing resistance to passage of the scope. He was then admitted to our hospital and underwent a detailed medical examination and treatment.

History of past illness

He had no specific past illness but had a current active smoking status [Brinkman Index: 920 (20 × 46 years)].

Personal and family history

No family history to note.

Physical examination

Mild tenderness was noted in the upper abdomen.

Laboratory examinations

Initial laboratory data revealed a hemoglobin level of 11.0 g/dL, white blood cell count of 9700 cells/μL, and platelet count of 3.17 × 10^5/μL. The creatinine level was 0.81 mg/dL, total bilirubin level was 0.3 mg/dL, direct bilirubin level was 0.1 mg/dL, aspartate aminotransferase level was 43 IU/L, alanine aminotransferase level was 72 IU/L, and albumin level was 3.5 g/dL. Tumor marker level of the carcinoembryonic antigen was 23.00 ng/mL, and carbohydrate antigen 19-9 level was 53.20 U/mL.

Imaging examinations

EGD identified stenosis caused by a large tumor (Figure 1). Computed tomography (CT) showed lymph node (LN) metastases at the station of the lesser curvature (#3 LN; 11.8 mm × 8.5 mm, Figure 2A), right greater curvature nodes along the right gastroepiploic artery (#4d LN; 10.3 mm × 8.4 mm, Figure 2B), infrapyloric nodes (#6 LN; 21.6 mm × 14.7 mm, Figure 2C), anterosuperior LNs along the common hepatic artery (#8a; 14.0 mm × 13.4 mm, Figure 2D), and suspicion of metastatic #6 LN invasion to the pancreatic head (the names of the LN station are provided in Table 1). There were no findings of distant metastasis.

Biopsies were taken, and the histological examination led to a diagnosis of adenocarcinoma (papillary and well-differentiated adenocarcinoma; Figure 3A). Additional pathological examination revealed human epidermal growth factor receptor 2 (HER2) positivity based on an immunohistochemical score of 3 + (Figure 3B).

The clinical diagnosis was gastric cancer LD circ cType3 cT4b (panc) N2M0 cStageIVA according to the Union for International Cancer Control Tumor, Node Metastasis Classification of Malignant Tumors, Eighth Edition[14]. The lymph node station was defined according to the Japanese Classification of Gastric Cancer, 15th Edition[15].

FINAL DIAGNOSIS

Pathological examination and classification

Pathological examination of the resected specimen revealed that the primary tumor was mixed-type adenocarcinoma (papillary adenocarcinoma, moderately differentiated adenocarcinoma > poorly differentiated adenocarcinoma non-solid type, mucinous adenocarcinoma) and subserosal invasion (35 mm × 30 mm) with lymphatic

Table 1 Lymph node station

Station No.	Definition
1	Right paracardial nodes
3	Lesser curvature nodes
4sb	Left greater curvature nodes along the left gastroepiploic artery
4d	Right greater curvature nodes along the right gastroepiploic artery
5	Suprapyloric nodes
6	Infrapyloric nodes
7	Nodes at the root of the left gastric artery
8a	Anterosuperior LNs along the common hepatic artery
8p	Posterior LNs along the common hepatic artery
9	Nodes at the celiac artery
11p	Nodes along the proximal splenic artery
12a	Hepatoduodenal ligament LNs along the proper hepatic artery
12b	Hepatoduodenal ligament LNs along the bile duct
12p	Hepatoduodenal ligament LNs along the portal vein
14v	Nodes along the superior mesenteric vein
13a	Superior posterior pancreatoduodenal lymph nodes
13b	Inferior posterior pancreatoduodenal lymph nodes
17a	Superior anterior pancreatoduodenal lymph nodes
17b	Inferior anterior pancreatoduodenal lymph nodes

LNs: Lymph node.

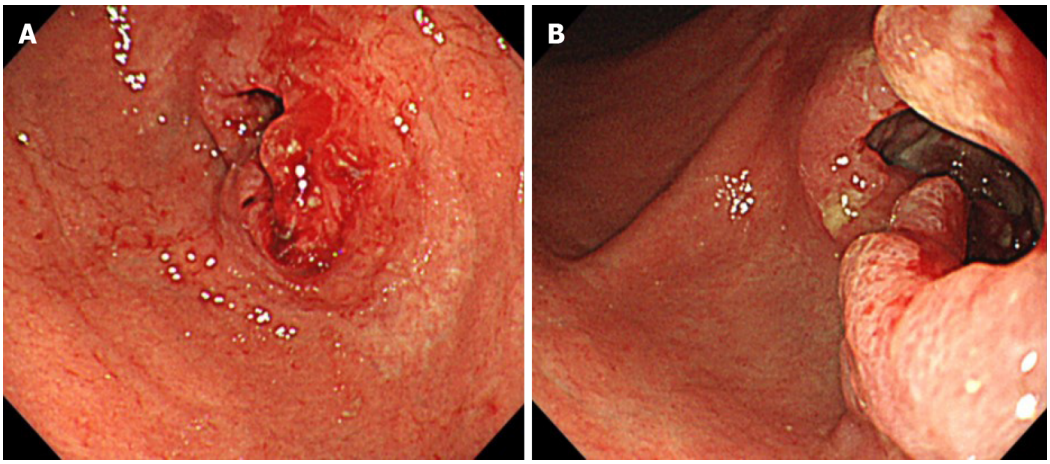

Figure 1 Initial endoscopic findings. A and B: Type 3 primary tumor located on the lower part of the stomach.

and venous infiltration (Ly1a, V1a). The horizontal and vertical surgical margins were negative, and all dissected LNs were negative for metastasis (0/54). Pathological findings also suggested pancreatic infiltration. The cancer cells infiltrating the pancreas may have invaded along the lymph nodes or nerves on the surface of the pancreas. (Figure 4). The pathological diagnosis was gastric cancer LD Circ ypType3, ypT4b (panc) N0M0, ypStage IIIA. Degeneration of cancer cells was limited, and the pathological therapeutic effect was classified as grade 1a according to the Japanese Classification of Gastric Cancer, 15th Edition[15].

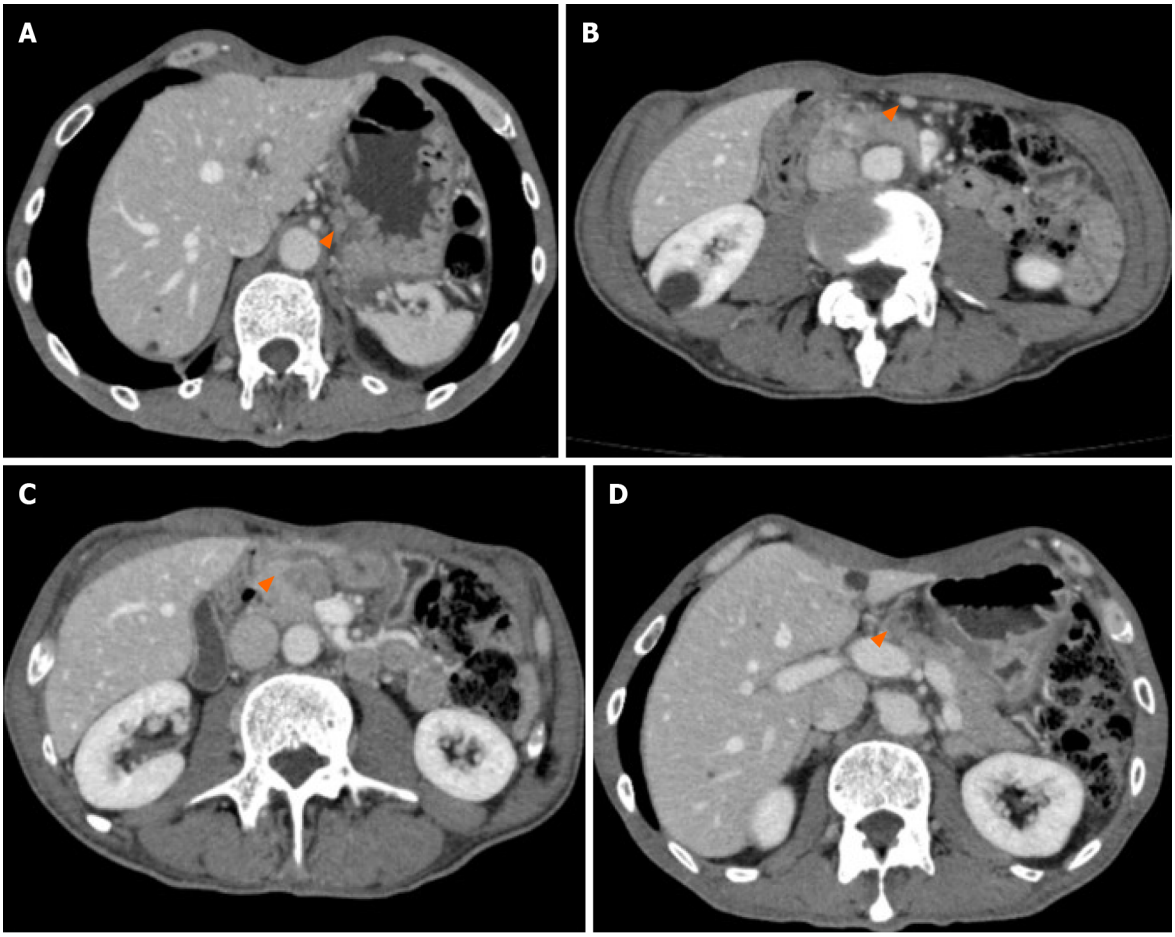

Figure 2 Initial computed tomography findings, computed tomography showed lymph node swelling. A: Swelling of #3 lymph node (LN) (orange triangle; 11.8 mm × 8.5 mm); B: Swelling of #4d LN (orange triangle; 10.3 mm × 8.4 mm); C: Swelling of #6 LN, suspected to be invasion to the pancreatic head (orange angle; 21.6 mm × 14.7 mm); D: Swelling of #8a LN (orange triangle; 14.0 mm × 13.4 mm).

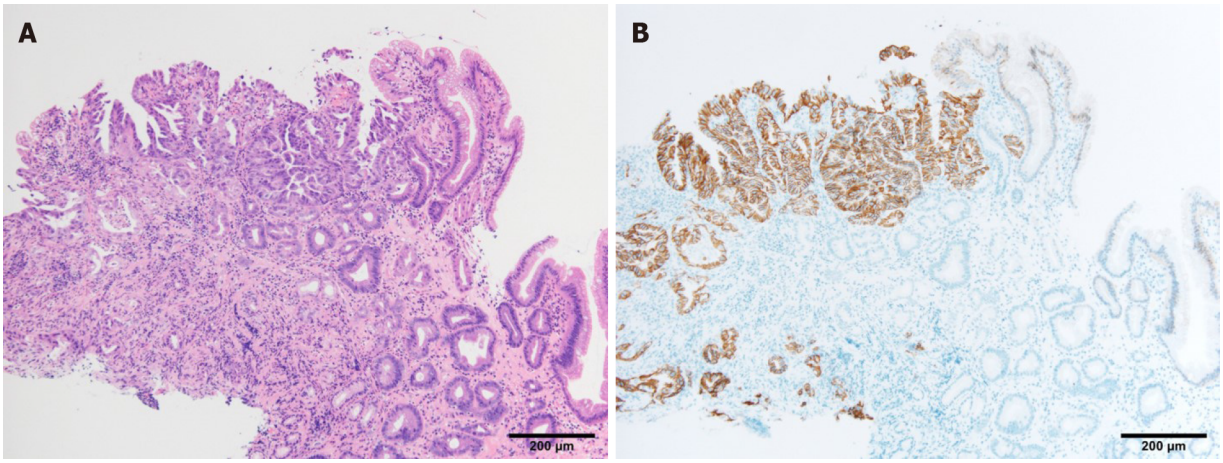

Figure 3 Pathological findings from endoscopic biopsy. Pathological examination of the endoscopic biopsy revealed a papillary and well-differentiated adenocarcinoma. A: Hematoxylin and eosin staining results; × 10; B: Human epidermal growth factor receptor 2 positivity was detected by an immunohistochemical staining; × 10.

TREATMENT

As the patient was undernourished, we inserted a double elemental diet tube to administer enteral nutrition and drain food residue from the stomach. After 10 d of continuous enteral nutrition, we performed staging laparoscopy and confirmed no liver metastasis, no dissemination, and negative lavage cytological results. In addition,

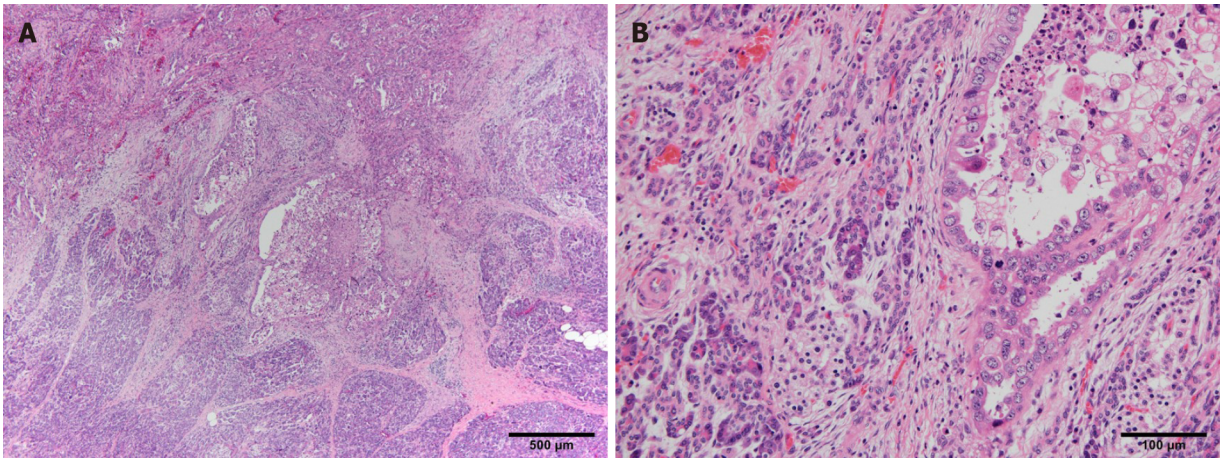

Figure 4 Pathological findings from resected specimens showing pancreatic infiltration of cancer cells. A: Hematoxylin and eosin staining results; × 4; B: Hematoxylin and eosin staining results; × 20.

immobility of the lower part of the stomach body due to cancerous invasion to the pancreatic head was confirmed. Thus, laparoscopic ante-colic gastrojejunostomy (GJ) and half-cut of the gastric body at the distal side of the anastomosis were performed.

Considering the clinical and intraoperative findings, it was a locally advanced gastric cancer with pancreatic head invasion without non-curative factors such as dissemination and distant metastasis. As a treatment strategy, preoperative chemotherapy was given first, and in the case of stable disease or partial response, R0 resection with PD could be adopted.

Neoadjuvant chemotherapy and toxicities

After laparoscopic GJ, one course of SOX (S-1 120 mg/d on days 1-14, oxaliplatin 100 mg/m^2 on day 1) was performed during one continuous hospitalization stay after surgery, and three courses of SOX with trastuzumab (S-1 120 mg/d on days 1-14, oxaliplatin 100 mg/m^2 on day 1, trastuzumab 8 mg/kg during the first cycle and 6 mg/kg during the second and third cycles) was performed in an outpatient setting. For this regimen, only grade 2 elevations in aspartate aminotransferase and alanine aminotransferase levels (84 and 96 IU/L, respectively) were observed, and no grade 3 or more severe adverse events were detected according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 5.0[16].

Radiological evaluation

After neoadjuvant chemotherapy, EGD showed shrinkage of the primary tumor, but the scope could not pass through (Figure 5A and B), while CT showed marked reduction of the primary tumor and metastatic LNs. The #3 (Figure 6A) and #4d (Figure 6B) LNs could not be detected, and #6 LN, which had been suspected of invading the pancreatic head before chemotherapy, was scarred and could not be measured (Figure 6C). The #8a LN had shrunk (from 14.0 mm × 13.4 mm to 9.2 mm × 7.0 mm; Figure 6D). The clinical therapeutic effect was classified as a partial response on radiological examination according to the Response Evaluation Criteria in Solid Tumors criteria version 1.0.

Although the invasion of the pancreas was not clear on CT, magnetic resonance imaging showed that most of the boundary between the stomach or lymph nodes around the stomach and pancreas had fatty tissue, but some of the boundaries were unclear; thus, the invasion could not be denied. Thus, the preoperative diagnosis was ycT4b (panc) N1M0 ycStage IVA.

Preoperative medical examination

The surgical risks for this patient included his age (66 years), obstructive pulmonary dysfunction [vital capacity (%), 101.5; forced expiratory volume in 1 s (%), 69.5; forced expiratory volume in 1 s (L), 2.48], current heavy smoker status [Brinkman Index: 920 (20 × 46 years)], undernutrition (total protein level 5.8 g/dL, albumin level 3.2 g/dL), a history of GJ, and the patient's clinical state after four courses of neoadjuvant chemotherapy. Cardiac function was normal (normal wall motion, ejection fraction of 72.8%) after neoadjuvant chemotherapy with trastuzumab, and the brain natriuretic

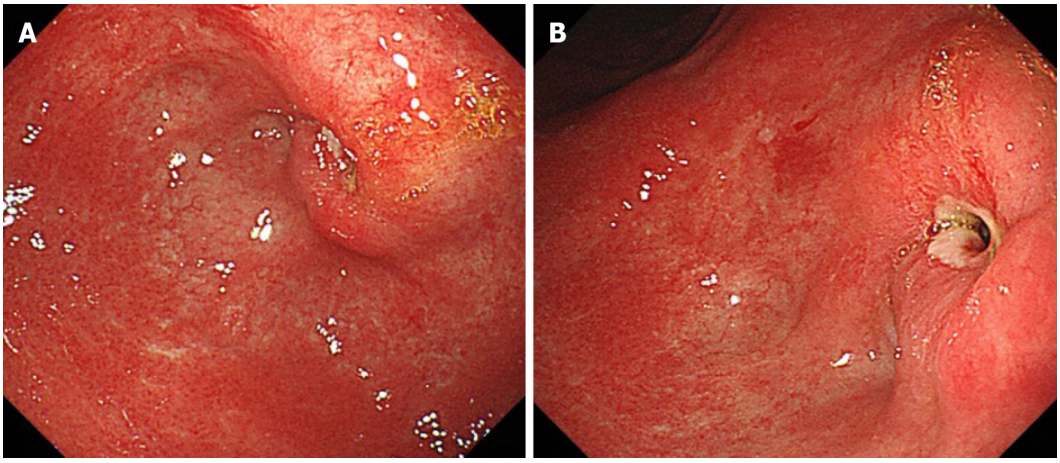

Figure 5 Endoscopic findings after neoadjuvant chemotherapy. A and B Shrinkage of the primary tumor.

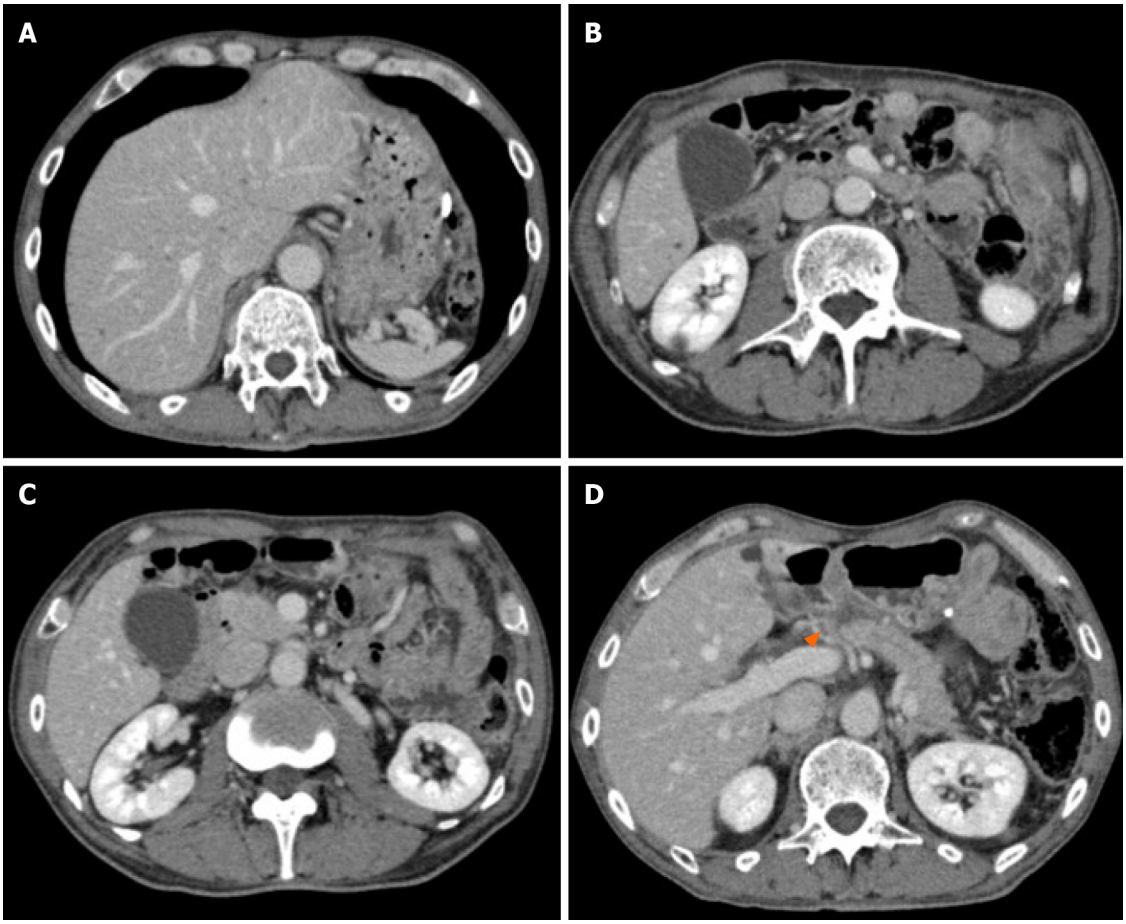

Figure 6 Computed tomography findings after neoadjuvant chemotherapy. A: #3 lymph node (LN) became smaller and could not be detected; B: #4d LN became smaller and could not be detected; C: #6 LN became smaller and could not be detected; D: #8a LN became smaller (orange triangle; 14.0 mm × 13.4 mm to 9.2 mm × 7.0 mm).

peptide level was 33.3 pg/mL. Tumor marker levels were decreased compared with initial blood tests, and the carcinoembryonic antigen level improved from 23.00 to 11.00 ng/mL and the carbohydrate antigen 19-9 level from 53.20 to 26.80 U/mL. The therapeutic strategy was explained to the patient, who decided to undergo surgical treatment.

Pancreaticoduodenectomy with D2 lymph node dissection for distal gastric cancer

After detachment of adhesions between the omentum and abdominal wall, we

confirmed no liver metastasis or dissemination. Rapid lavage cytology revealed no cancer cells in the ascites (Class I). We first performed omentectomy to expose the layer of the colic vein and superior mesenteric vein and performed Kocher mobilization until the inferior vena cava could be identified; the layers were continued up to the layer of the omentectomy. Near the right gastroepiploic artery and vein, the metastatic LN and/or tissues with desmoplastic and inflammatory reactions near the primary lesion invaded the pancreatic head and could not be separated. Therefore, PD was required to achieve R0 resection. We made a jejunum incision at the proximal and distal sides of the anastomosis, which was bypassed in a previous surgery, and performed D2 lymphadenectomy (#1, #3a, #3b, #4sb, #4d, #5, #6, #7, #8a, #9, #11p, #12a) with #8p and #14v LN dissection. The gastric body was cut using an Endo GIA 60 mm stapler (Covidien, Mansfield, MA, United States). After regional LN dissection of the gastric cancer, the gastroduodenal artery was ligated. The dorsal side of the pancreas was tunneled in front of the portal vein. We decided that resection of the tumor could be performed if the pancreas was cut at the line just on the portal vein.

The vein branches of the superior mesenteric vein (first jejunal vein, inferior pancreaticoduodenal vein, gastrocolic trunk, posterior superior pancreatoduodenal vein) were processed, and the artery branches (first jejunal artery, inferior pancreaticoduodenal artery, dorsal pancreatic artery) were processed outside the plexus of the superior mesenteric artery. After excision of the gallbladder, the common bile duct was dissected, and PD was completed (#13a, #13b, #17a, #17b, #12b, and #12p were taken together with the specimen).

Reconstruction

Reconstruction of modified Child's method with Braun enteroenterostomy was performed. First, pancreaticojejunostomy and cholangiojejunostomy were performed, followed by antecolic reverse peristaltic GJ. Jejunum of the afferent loop was sutured to the gastric body to prevent reflux to the afferent loop. Finally, jejunostomy was performed distal to the GJ, and Braun enteroenterostomy was made. The total operation time was 11 h and 16 minutes, and total blood loss was 490 mL.

OUTCOME AND FOLLOW-UP

On the day after the operation, the patient started drinking water and using a jejunostomy tube for enteral nutrition, and he resumed eating on the second day. Surgical complications included aspiration pneumonia, pancreatic fistula, and intraabdominal abscess, each of which was cured with antibiotics and defined as Clavien-Dindo grade 2[17]. The patient was discharged on postoperative day 16 without the need for enteral nutrition. Adjuvant S-1 (100 mg/body) was started 2 mo after the surgery and SOX (S-1; 120 mg/d on days 1-14, oxaliplatin; 100 mg/m^2 on day 1) was started 3 mo after the surgery. Postoperative SOX chemotherapy was performed for three courses. There are no recurrences 8 mo after surgery. The timeline was showed in Table 2.

DISCUSSION

We present the successful case of radical resection with PD after neoadjuvant chemotherapy combined with trastuzumab for HER2-positive locally advanced gastric cancer invading the pancreatic head.

The principle of surgical oncology with curative intent is *en bloc* resection of organs potentially invaded by the cancer. In cases of pancreatic head invasion, PD with gastrectomy is required. Because of the high surgical risk and poor prognosis, PD is rarely performed for gastric cancer. However, due to advances in surgery and improvements in perioperative management, PD may be considered for gastric cancer to improve long-term survival. Recent reports from two Japanese high-volume cancer centers reported morbidity rates of 45.1% (14/31) and 73.9% (17/23) and mortality rates of 0% and 12.9% (4/31), respectively, after PD for gastric cancer[5,7]. Pancreatic fistula was the most common complication in these studies (12.9% and 43.5%). Saka *et al*[7] reported that early detection of pancreatic fistulas and drain discharge management are important to prevent fatal complications, such as an intraabdominal abscess leading to rupture of pseudoaneurysms, and emphasized that medical staff (including the surgeon and nursing staff) should be trained in drain management.

Table 2 Timeline from the onset of symptoms to the completion of treatment

Time series	Symptoms and treatment details
5 mo ago	He had symptoms of abdominal pain, distension and weight loss (from 62 to 47 kg within 6 mo) and visited hospital
	Double elemental diet tube was inserted to administer enteral nutrition and drain food residue from the stomach
4 mo ago	Staging laparoscopy confirmed no liver metastasis, no dissemination, and negative lavage cytological results
	Laparoscopic ante-colic gastrojejunostomy and half-cut of the gastric body at the distal side of the anastomosis were performed
1-4 mo ago	SOX 1
	SOX + Trastuzumab 2-4
The date of surgery	Pancreaticoduodenectomy with D2 lymph node dissection for distal gastric cancer was done
	Adjuvant S1 1
2-8 mo after surgery	Adjuvant SOX 1-3
	CT shows no recurrence (7 mo after surgery)

CT: Computed tomography; SOX: S-1 plus oxaliplatin.

Therefore, those authors recommended that PD should be performed only at institutions comfortable with PD management.

In our institution, PD is typically performed for pancreatic cancer by hepatobiliary-pancreatic surgeons and appropriate drainage treatment can be performed by radiologists if necessary. Thus, in this case, upper gastrointestinal surgeon and hepatobiliary-pancreatic surgeon performed the surgery in collaboration, allowing us to perform adequate regional LN dissection of the stomach and provide safe surgery and postoperative management for PD. In the present case, although severe inflammatory reactions around the tumor and degeneration and desmoplastic changes due to neoadjuvant chemotherapy were observed, the intraoperative blood loss was 490 mL, blood transfusion was not required, the intensive care unit stay was 1 d, postoperative complications did not exceed grade 2, and the patient was discharged 16 d after surgery. In addition, from an oncological perspective, R0 resection was achieved, and the number of LNs dissected was 54.

Li *et al*[8] reported a 5-year overall survival (OS) rate of gastric cancer patients after PD of 39.3%, which is comparable with that of patients with stage IIIB (34.8%) gastric cancer, as is registered nationally by the Japanese Gastric Cancer Society[18]. Katai *et al*[18] also reported, in a nationwide study, a rate of PD performed for gastric cancer of 0.1% (120/12202) and a 5-year OS rate of 41.4%. These findings suggest that PD is a beneficial technique under the correct indications. Saka *et al*[7] reported that the 5-year OS rate was 0% in patients with non-curative factors [such as para-aortic LN (PAN) metastasis, positive lavage cytological results, and peritoneal dissemination] and 47.4% in patients those without these factors. Nunobe *et al*[5] reported that the 5-year OS rate after R0 resection with PD was significantly better in the ≤ pN2 group (50.0%) compared with the ≥ pN3 group (7.7%). In our case, we confirmed negative lavage cytological results, no PAN, no dissemination during surgery, and no detected LN metastasis pathologically after surgery. All of these factors may affect survival after PD. According to these studies, PD is considered to be required in gastric cancer surgery in about 1 in 500 to 1000 cases[7,18], of which about one-third may have incurable factors[7]. Combining neoadjuvant chemotherapy may reduce the proportion of incurable cases and increase the rate of radical resection with PD.

Several randomized studies have shown a survival benefit of postoperative adjuvant chemotherapy for advanced gastric cancer[9-11]. However, no reports have described the efficacy or completion rate of adjuvant chemotherapy after PD. The completion rate of adjuvant chemotherapy after routine gastrectomy was reported to be 65.8% for S-1[9], 67% for capecitabine plus oxaliplatin[10], and 49% for docetaxel plus S-1[11]. This indicates that 33%-51% of patients do not benefit from chemotherapy. Adjuvant chemotherapy may be inadequate if the procedure becomes more invasive because of major surgical complications combined with organ resection and loss of appetite or physical fitness. Thus, preoperative chemotherapy is considered to have benefits in these patients compared with postoperative chemotherapy. Clinical trials of neoadjuvant chemotherapy for gastric cancer with extensive nodal metastasis (bulky and/or PANs) reported high completion rates of 65%-81%[19-21]. Despite the inclusion of

double or triple chemotherapy regimens in these trials, compliance was greater compared with single-agent postoperative adjuvant chemotherapy. Considering the effects of preoperative chemotherapy and the highly invasive postoperative situation, patients with locally advanced gastric cancer who require PD for radical resection are thought to be good candidates for neoadjuvant chemotherapy. We used SOX plus trastuzumab as preoperative chemotherapy because, at our facility, we typically use the SOX regimen as preoperative chemotherapy for advanced gastric cancer with multiple LN metastases, according to a recent ongoing randomized clinical trial (JCOG1509)[13]. Furthermore, preoperative and perioperative chemotherapies combined with trastuzumab for HER2-positive gastric cancer are currently underway[12,22]. Thus, we think that SOX plus trastuzumab combination is expected as effective preoperative chemotherapy for HER-2 positive advanced gastric cancer.

Another problem is that invasion of adjacent organs cannot always be confirmed pathologically[7]. Desmoplastic and inflammatory reactions surrounding the tumor cannot be differentiated from tumor invasion during surgery; thus, PD cannot be avoided in such cases. However, neoadjuvant chemotherapy has a possibility to differentiate between cases of true tumor invasion and inflammatory reactions and may even avoid PD in some cases.

There were some limitations to this case report. First, the long-term survival benefit of neoadjuvant chemotherapy combined with trastuzumab in this patient was unknown, and a longer observation time is required. However, we achieved R0 resection with PD after this regimen and confirmed no LN metastasis pathologically. Previous reports have reported a survival benefit in such cases. Second, we use SOX combined with trastuzumab, for which no clear evidence has been established. However, the therapeutic effects of neoadjuvant SOX (JCOG1509; UMIN000024065) and neoadjuvant chemotherapy combined with trastuzumab for advanced gastric cancer (JCOG1301; UMIN000016920[12]) are currently being verified in clinical trials in Japan.

CONCLUSION

We suggest that radical resection with PD after neoadjuvant chemotherapy combined with trastuzumab is one option for locally advanced HER2-positive gastric cancer invading the pancreatic head in the absence of non-curative factors.

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