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*World Journal of Gastroenterology* (*WJG*) is now indexed in Current Contents<sup>®</sup>/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch<sup>®</sup>), Journal Citation Reports<sup>®</sup>, Index Medicus, MEDLINE, PubMed, PubMed Central and Directory of Open Access Journals. The 2017 edition of Journal Citation Reports<sup>®</sup> cites the 2016 impact factor for *WJG* as 3.365 (5-year impact factor: 3.176), ranking *WJG* as 29<sup>th</sup> among 79 journals in gastroenterology and hepatology (quartile in category Q2).

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**NAME OF JOURNAL**  
*World Journal of Gastroenterology*

**ISSN**  
ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

**LAUNCH DATE**  
October 1, 1995

**FREQUENCY**  
Weekly

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Baishideng Publishing Group Inc  
7901 Stoneridge Drive, Suite 501,  
Pleasanton, CA 94588, USA  
Telephone: +1-925-2238242  
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Help Desk: <http://www.f6publishing.com/helpdesk>  
<http://www.wjgnet.com>

**PUBLICATION DATE**  
January 21, 2018

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## Use of direct-acting antiviral agents in hepatitis C virus-infected liver transplant candidates

Chiranjeevi Gadiparthi, George Cholankeril, Brandon J Perumpail, Eric R Yoo, Sanjaya K Satapathy, Satheesh Nair, Aijaz Ahmed

Chiranjeevi Gadiparthi, Sanjaya K Satapathy, Satheesh Nair, Division of Gastroenterology and Hepatology, University of Tennessee Health Sciences Center, Memphis, TN 38104, United States

George Cholankeril, Aijaz Ahmed, Division of Gastroenterology and Hepatology, Stanford University School of Medicine, Stanford, CA 94304, United States

Brandon J Perumpail, Drexel University College of Medicine, Philadelphia, PA 19129, United States

Eric R Yoo, Department of Medicine, Santa Clara Valley Medical Center, San Jose, CA 95128, United States

ORCID number: Chiranjeevi Gadiparthi (0000-0002-8905-6742); George Cholankeril (0000-0001-5335-8426); Brandon J Perumpail (0000-0001-7716-6824); Eric R Yoo (0000-0002-0584-6975); Sanjaya K Satapathy (0000-0003-0153-2829); Satheesh Nair (0000-0002-4167-6643); Aijaz Ahmed (0000-0002-3609-8586).

**Author contributions:** All authors contributed to study concept and design, acquisition of data, analysis and interpretation of data, drafting, editing, and critical revision of manuscript and, and approval of final version.

**Conflict-of-interest statement:** The authors have no conflict of interest related to this publication.

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**Manuscript source:** Invited manuscript

**Correspondence to:** Aijaz Ahmed, MD, Associate Professor, Attending Doctor, Division of Gastroenterology and Hepatology,

Stanford University School of Medicine, 750 Welch Road, Suite 210, Palo Alto, CA 94304, United States. [aijazahmed@stanford.edu](mailto:aijazahmed@stanford.edu)  
Telephone: +1-650-4986091  
Fax: +1-650-4985692

**Received:** November 7, 2017

**Peer-review started:** November 7, 2017

**First decision:** November 30, 2017

**Revised:** December 5, 2017

**Accepted:** December 12, 2017

**Article in press:** December 12, 2017

**Published online:** January 21, 2018

### Abstract

Since the advent of direct acting antiviral (DAA) agents, chronic hepatitis C virus (HCV) treatment has evolved at a rapid pace. In contrast to prior regimen involving ribavirin and pegylated interferon, these newer agents are highly effective, well-tolerated, have shorter course of therapy and safer essentially in all HCV patients including those with advanced liver disease and following liver transplantation. Clinicians caring for HCV-infected patients on the liver transplant (LT) waitlist are often faced with a dilemma whether to treat HCV infection before or after liver transplantation. Sustained virological response (SVR) rates following HCV treatment may improve hepatic function sufficiently enough to negate the need for LT in certain patients. On the other hand, the decrease in MELD without improvement in quality of life in certain patients may lead to delay or dropout from potentially curative LT surgery list. In this context, our review focuses on the approach to and optimal timing of DAA-based treatment of HCV infection in LT candidates in the peri-transplant period.

**Key words:** Hepatitis C virus; Direct-acting antiviral therapy; Liver transplantation; Purgatory Model for End-stage liver disease; Sustained virological response



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**Core tip:** Optimal timing of antiviral therapy for hepatitis C virus (HCV) infection in liver transplant candidates using second generation direct-acting antivirals is debated. Available evidence lacks conviction if the viral eradication is beneficial in all HCV patients before liver transplantation. We aim to review the current literature to better delineate the appropriate timing of HCV treatment in the era of direct-acting antiviral agents.

Gadiparthi C, Cholankeril G, Perumpail BJ, Yoo ER, Satapathy SK, Nair S, Ahmed A. Use of direct-acting antiviral agents in hepatitis C virus-infected liver transplant candidates. *World J Gastroenterol* 2018; 24(3): 315-322 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i3/315.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i3.315>

## INTRODUCTION

Chronic hepatitis C virus (HCV) infection continues to be a major cause of chronic liver disease in the United States (US) despite the overall decline in the incidence in recent years. Based on current estimates, 2.7 to 3.5 million persons are infected with chronic HCV (1.0%-1.5% of US population) and HCV-related liver disease accounts for more than 15000 deaths annually<sup>[1-3]</sup>. With 30% of all adult liver transplant surgeries performed annually in patients with HCV-related end-stage liver disease, HCV continues to be the leading indication for liver transplantation (LT) in the US<sup>[4,5]</sup>. However, with the introduction of second generation direct-acting antiviral (DAA) agents five years ago, the paradigm of HCV treatment landscape has shifted dramatically. With a very favorable safety profile and high rates of sustained virological response (SVR) of over 95%, the newer and all-oral DAA-based regimens have provided an unprecedented opportunity to cure HCV. Although HCV-disease burden remains substantial at the moment, it is estimated that, within next decade, most patients with HCV in clinical practice will likely have attained SVR<sup>[6]</sup>. Furthermore, SVR can forestall the progression of liver disease with subsequent reduction in liver-related complications including hepatocellular carcinoma (HCC), hepatic decompensation, and both liver related as well as all-cause mortality<sup>[7]</sup>. Although the newer DAA-based therapy has been highly effective, the optimal timing of HCV treatment in LT candidates is unclear and remains a subject of much debate. Achieving SVR following a DAA-based therapy in LT candidates with HCV-related cirrhosis may result in decrease in the Model for End-Stage Liver Disease (MELD) score without a significant improvement in quality of life due to persistence of end-stage liver disease-related complications (a concept of 'MELD purgatory')<sup>[8,9]</sup>. Furthermore, the improvement in

MELD score may result in removal of these candidates from transplant waitlist thus reducing the likelihood of undergoing LT and potentially contributing to waitlist mortality. In addition to examining the validity of 'MELD purgatory', we seek to evaluate the current data pertaining to chronic HCV treatment in the context of liver transplantation.

## NATURAL HISTORY OF HCV PRIOR TO LIVER TRANSPLANTATION

HCV can cause both acute and chronic infections although the latter is more common. Within two weeks after exposure to HCV, up to 20% of patients develop acute hepatitis, which is often asymptomatic although a few may experience nausea, anorexia, malaise, and jaundice. Due to inability to spontaneously clear the virus, a clear majority of patients (approximately 55%-85%) develop chronic HCV infection. As a result of chronicity and slow progression, HCV leads to cirrhosis in 10%-40% of patients over a period of 20-30 years<sup>[10]</sup>. However, in certain populations such as those with HIV co-infection, elderly patients, and liver transplant recipients, a rapid progression to advanced stages of liver disease can occur<sup>[11]</sup>. Since most patients with chronic HCV infection are asymptomatic, the diagnosis of HCV is often delayed until after the development of cirrhosis or onset of an index complication. After the onset of cirrhosis, the rates of hepatic decompensation in these patients are 3%-6% and annual risk of HCC is 1%-5%<sup>[12]</sup>. The rates of decompensation and HCC are influenced by interplay of a variety of host and viral factors. HCV accounts for 55% of all HCC in cirrhosis patients currently, making it the leading cause of HCC in the US<sup>[13]</sup>. There is 15%-20% risk of mortality within one year after the development of hepatic decompensation, and LT usually serves as the only life-saving therapeutic option<sup>[14]</sup>.

## NATURAL HISTORY OF HCV IN LIVER TRANSPLANT RECIPIENTS

HCV-infected patients with end-stage liver disease with or without HCC have a clear survival advantage with LT, which serves as a curative therapy. However, in patients with pre-transplant viremia who do not receive HCV treatment prior to LT, post-transplant HCV recurrence is usually immediate and universal. HCV reinfection results in graft dysfunction with progression to cirrhosis in about one-third of patients within 5 years after LT compared to less than 5% in non-transplant patients<sup>[15]</sup>. Spontaneous viral clearance following LT is reported in a few published cases although the underlying mechanism is not clearly understood<sup>[16]</sup>. Nevertheless, HCV infection of allograft has protracted course with rapid progression and higher mortality risk compared to non-transplant chronic HCV infection.

The recurrent HCV infection is defined as presence of HCV RNA in serum and/or liver, however histological confirmation is required for establishing the recurrent disease<sup>[17]</sup>. Histopathological changes related to recurrent disease in the allograft are similar to those of an immunocompetent patient, usually develop within 3 months after the LT surgery, with 70%-90% of transplant recipients demonstrating the changes of chronic hepatitis at 1 year, and 90%-95% at 5 years<sup>[18]</sup>. Similarly, the rates of decompensation after onset of graft cirrhosis are > 40% and > 60% at 1 and 3 years respectively<sup>[19,20]</sup>, whereas in pre-transplant chronic HCV patients the rates are < 5% and < 20% at 1 and 5 years respectively<sup>[21,22]</sup>.

In pre-DAA era, due to poorly tolerated, ineffective interferon-based therapy and accelerated fibrosis, LT recipients with HCV had inferior graft and patient survival compared to those who underwent LT for non-HCV indications. In a large study using United Network for Organ Sharing (UNOS) data comparing 4439 HCV-positive and 6597 HCV-negative recipients, the 5-year graft and patient survival rates were found to be 57% and 70% in HCV-positive recipients compared with 68% and 77% respectively in HCV-negative counterparts<sup>[23]</sup>. However, with the availability of highly effective and safer DAA-based agents, HCV-positive LT recipients are expected to have outcomes similar to those undergoing LT for other indications<sup>[24,25]</sup>.

## TREATMENT OF HCV PRIOR TO LIVER TRANSPLANTATION

In pre-DAA era, pegylated interferon and ribavirin were cornerstone of HCV treatment. Prior studies have shown that achieving SVR by viral eradication with interferon-based treatment regimen has shown to significantly reduce the rates of cirrhosis, decompensation, HCC, and both liver-related and all-cause mortality<sup>[7,26]</sup>. However, interferon-based regimen was poorly tolerated due to high rates of adverse events, had poor clinical efficacy with low SVR rates and could not be used in patients with decompensated cirrhosis<sup>[27]</sup>. DAA-based therapy, on the other hand, is highly efficacious with high rates of SVR, well tolerated leading to better patient adherence, and can be used safely in patients with advanced liver disease<sup>[28]</sup>. Although these attributes can be compelling for clinicians to treat all chronic HCV patients, it is however important to understand the optimal timing of HCV therapy.

Recently, several studies have demonstrated very favorable results with DAAs in patients with advanced liver disease before and after LT. In SOLAR-1 trial in 2015, a combination of sofosbuvir, ledipasvir and ribavirin for 12-24 wk achieved high rates of SVR at 12 wk (SVR12) in patients with advanced liver disease, including those with decompensated cirrhosis both before and after LT<sup>[25]</sup>. In this landmark trial, SVR12 in non-transplant cohort with moderate to severe hepatic impairment [Child-Turcotte-Pugh (CTP) class B and

C] was 86%-89%, whereas the rates in transplant recipients were 96%-98% in mild hepatic impairment group (CTP A), 85%-88% in moderate hepatic impairment group (CTP B), and 60%-75% severe hepatic impairment group (CTP C). More importantly, among seven patients who required re-transplantation, including four patients who underwent LT prior to completion of HCV therapy, six patients achieved SVR 12 suggesting the high efficacy of DAAs in preventing the recurrence of HCV infection after LT.

DAAs have shown to be equally effective in different ethnic groups and certain high-risk HCV populations including elderly, and treatment experienced patients who failed prior therapies. In a multicenter, international ASTRAL-1 trial, a combination sofosbuvir and velpatasvir noted high efficacy rates in African-Americans and previously failed, treatment experienced HCV patients<sup>[29]</sup>. Similarly, ASTRAL-2 and ASTRAL-3 trials showed that HCV patients with genotype 2 and 3 infection achieved SVR rates of 99% and 95% respectively, which were superior to standard therapy with sofosbuvir and ribavirin which had SVR rate of 80%<sup>[30]</sup>. DAA agents are highly effective in elderly patients as well and are generally safe. In a retrospective study evaluating the safety and efficacy of ledipasvir/sofosbuvir with or without ribavirin, elderly HCV patients (age > 65 years) with genotype 1 infection achieved similar SVR rates (98%) compared to the younger subjects (97%), however treatment related adverse events that lead to discontinuation of therapy in elderly subjects was largely related to ribavirin rather than ledipasvir/sofosbuvir<sup>[31]</sup>. DAA-based therapy, through an expanded access program, in patients with decompensated cirrhosis (CTP B and C) who are at risk of irreversible disease achieved overall SVR of 81.6% (genotype 1, 90.5% and genotype 3, 68.8%)<sup>[32]</sup>. Importantly, within 6 months following viral clearance, 60% patients noted improvement in hepatic function while 17% had no change and 23% had worsening MELD score.

These major trials have repeatedly demonstrated that DAA-based therapy is not only efficacious but well tolerated across the wide spectrum of patients with HCV-related liver disease including those with advanced and decompensated cirrhosis. Data from integrated safety analysis of SOLAR 1 and 2 trials showed that a combination of sofosbuvir and ledipasvir with ribavirin in decompensated liver cirrhosis patients was generally safe and well-tolerated and major adverse events noted in 28%-30% patients and death in 5%<sup>[33]</sup>. Although all the major studies had enrolled HCV-patients with decompensated cirrhosis, it is important to note that the proportion of patients with higher MELD scores (> 20) and CTP-C was very low. Therefore, these results should be applied with caution in patients with severe hepatic impairment (higher MELD scores).

While all HCV patients can and should be treated, it may not be beneficial in patients for whom LT is the only curative option, for example, those with advanced liver disease or those with HCC. Additionally,

**Table 1** Advantages and disadvantages of hepatitis C virus treatment in liver transplant candidates before liver transplantation

Advantages	Disadvantages
1. Liver function and MELD score may improve	1. MELD score may improve but with ongoing poor health (MELD purgatory)
2. Liver transplantation may no longer be necessary	2. Possibly eliminates the option of a curative treatment for liver disease
3. Societal benefits given the scarcity of organs and limited donor pool	3. May limit access to HCV-positive donors, thereby prolonging the transplant waitlist time and risk of dropout or death
4. Prevent post-transplant recurrence of HCV	4. If treatment fails, risk of resistance to NS5A inhibitors and compromised SVR rates when re-treating after liver transplantation
5. Cost savings if liver transplantation can be obviated	

HCV: Hepatitis C virus; SVR: Sustained virological response; MELD: Model for end-stage liver disease.

after achieving viral clearance before LT, HCV patients are no longer able to accept the organs from HCV-positive donors, further shrinking the already limited donor pool. This is particularly relevant in midst of opioid epidemic, where the young and otherwise healthy HCV-positive donors are becoming increasingly available due to deaths related to overdose. In regions with high HCV prevalence, HCV treatment prior to LT may potentially extend the transplant wait period and thus increasing the risk of waitlist dropout due to inability to accept the offer from HCV-positive donors. Therefore, the decision of HCV treatment before or after LT largely depends on proportion of HCV-positive donors in the local and regional areas<sup>[34]</sup>. If the proportion of HCV-positive donor is sufficiently high, it may be beneficial to wait and treat HCV infection after transplantation. However, in future, policies may change necessitating the uniform acceptance of HCV-positive donors by all patients with or without HCV.

## COST-EFFECTIVENESS OF HCV TREATMENT

HCV patients who are already treated and cured prior to LT can still accept HCV-positive donors, however, these patients require re-treatment for recurrent HCV infection after transplantation incurring additional health-care costs associated with generally expensive DAA agents. But, the cost of HCV treatment should be examined in the context of overall costs associated with LT surgery, which is several fold higher than HCV therapy. In addition to mortality benefit, substantial cost-savings can be obtained as a result of reduction in the hospitalizations related to complications and need for transplantation<sup>[35]</sup>. Younossi *et al*<sup>[36]</sup>, showed that number needed to treat with DAAs to prevent one LT in patients with compensated cirrhosis is 15, which is quite remarkable. However, this study does not include the patients with advanced liver disease or decompensated cirrhosis. Results from another European study involving LT candidates demonstrated that following the DAA therapy, one out of five patients on transplant waitlist were successfully delisted due to significant clinical improvement<sup>[37]</sup>. Nevertheless, in patients with severe hepatic dysfunction and HCC in whom the LT cannot be avoided, HCV treatment

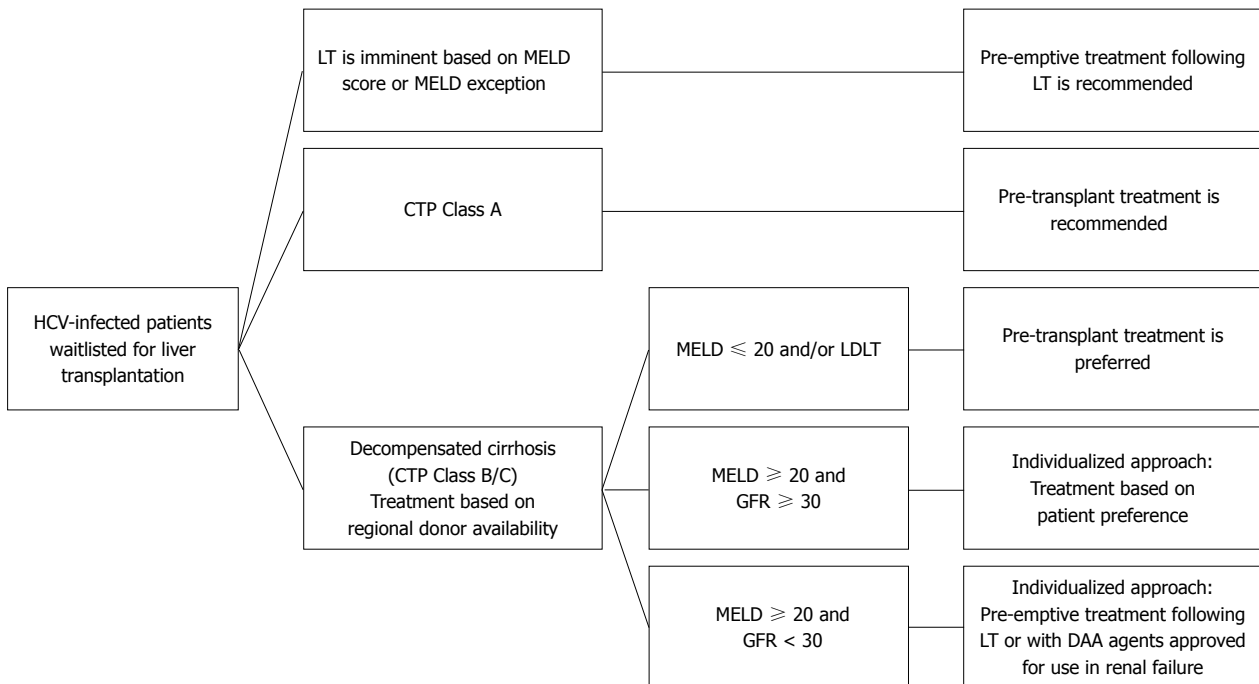
prior to LT may not be beneficial or cost-effective<sup>[35]</sup>. Advantages and disadvantages of this treatment strategy are summarized (Table 1).

## TREATMENT OF HCV FOLLOWING LIVER TRANSPLANTATION

HCV patients who are viremic at the time of transplantation almost always develop recurrent HCV infection after LT. Treatment of recurrent HCV infection in LT recipients followed by viral eradication results in significant improvement in post-transplant mortality and morbidity<sup>[38]</sup>. Prior to the approval DAAs, standard of care for recurrent HCV infection was based on pegylated interferon and ribavirin, which were poorly tolerated and had sub-optimal success. A systematic review by Berenguer *et al*<sup>[39]</sup>, involving 19 studies and total 611 LT recipients with recurrent HCV infection that were treated with interferon-based therapy, reported low rate of SVR (30.2%) due to significant adverse effects that lead to reduction in dose in 73% patients and discontinuation of therapy in 27.6%<sup>[39]</sup>. In contrast, DAA-based therapy is well tolerated and efficacious with high rates of SVR in LT recipients. Sofosbuvir plus ribavirin for 24 wk was first all-oral DAA regimen that was used in LT recipients which achieved SVR12 rate of 70%<sup>[40]</sup>. The combination of Sofosbuvir and simeprevir in several clinical trials demonstrated better tolerability and improved efficacy. This combination achieved SVR12 in 88% of LT recipients, however the rate was lower (64%) in LT recipients with advanced fibrosis<sup>[41,42]</sup>. Recently, in ALLY-1 study that included 55% LT recipients with advanced fibrosis, a combination of sofosbuvir, daclatasavir, and ribavirin for 12 wk was well-tolerated and achieved high SVR rate (91%-95%) across different genotypes<sup>[43]</sup>. Although all DAAs are generally safe and approved in post-transplant setting, attention must be paid to drug-drug interaction especially with immunosuppressive agents. For example, simeprevir is contraindicated in LT recipients who are on cyclosporine and ritonavir can increase the tacrolimus level 57 to 68-fold and cyclosporine level 4.3 to 5.8-fold<sup>[44]</sup>. Therefore, careful selection of DAA agents is warranted based on the patient's immunosuppressive therapy.

Fibrosing cholestatic hepatitis (FCH) is a severe





**Figure 1** A pragmatic treatment approach in hepatitis C virus-infected liver transplant candidates. HCV: Hepatitis C virus; MELD: Model for End-Stage Liver Disease; LT: Liver transplantation; CTP: Child-Turcotte-Pugh; GFR: Glomerular filtration rate; LDLT: Living donor liver transplantation.

form of HCV recurrence that results in progressive hepatic dysfunction and graft failure which was associated with high mortality in interferon era. However, in DAA era, the outcomes associated with FCH patients have been encouraging. In a compassionate use program, treatment of FCH patients with sofosbuvir and simeprevir achieved SVR12 of 80%<sup>[45]</sup>. Despite the absence of large prospective studies, the available evidence favors early treatment of recurrent HCV in post-transplant setting before the onset of fibrosis to achieve improved patient and graft survival.

### CONCEPT OF 'MELD PURGATORY'

Current LT allocation algorithm employs MELD score based prioritization, where sicker patients with higher MELD score are transplanted first. DAA-based therapy with viral eradication in candidates on LT waitlist may improve MELD score and hepatic dysfunction sufficiently enough that they may no longer require LT. This is an ideal scenario. However, some patients may experience decrease in MELD score without significant improvement in quality of life or hepatic dysfunction that puts them at risk of waitlist dropout or death- 'MELD purgatory'. These patients perhaps are best served by deferring the HCV treatment until after LT. A European study demonstrated that after achieving SVR following HCV treatment, 1 out of 5 candidates were removed from LT waitlist due to significant reversal of hepatic dysfunction<sup>[37]</sup>. More recently, another study from Spain showed that about a quarter of patients with decompensated cirrhosis were successfully removed from LT waitlist due to clinical improvement<sup>[46]</sup>. However, both

studies demonstrated that the patients with MELD > 20 were less likely to be removed from waitlist due to inadequate clinical or biochemical improvement. Thus, algorithms for HCV treatment have been proposed to avoid 'MELD purgatory'-authors recommended HCV treatment in patients with hepatic decompensation and MELD < 20 and scheduled for living donor LT<sup>[47]</sup>. However, patients with MELD 20-27 can be treated depending on the regional trends. In patients with MELD > 27 and/or severe kidney dysfunction (GFR < 30) should be treated after LT<sup>[47]</sup>. The safety and efficacy of DAAs in chronic kidney disease patients (GFR < 30) and those on hemodialysis is unknown. A few recent single center studies involving HCV patients with advanced chronic kidney disease (GFR < 30) or those on hemodialysis have shown favorable results with DAA agents in terms of safety and SVR rates compared to general population<sup>[48,49]</sup>. Although, these are small and single center studies, the preliminary results are encouraging, and evidence regarding DAA therapy in renal failure patients continues to emerge.

Finally, if transplantation is imminent and in the setting of severe hepatic impairment, it is prudent to wait and treat HCV infection after transplantation. Nonetheless, DAA-based therapy is beneficial and recommended in patients with mild hepatic impairment and select patients with moderate to severe hepatic impairment.

To avoid 'MELD purgatory', we suggest a modified algorithm (Figure 1) summarizing the approach to optimal timing of HCV therapy in LT candidates. At present, evidence favors DAA-based HCV therapy in patients with lower MELD scores and mild hepatic

impairment in pre-transplant period. Additionally, with exception to those needing imminent LT, carefully selected patients with moderate hepatic decompensation may also benefit from HCV therapy prior to transplantation.

## CONCLUSION

The advent of DAA agents has revolutionized the HCV treatment landscape. Due to their safety profile, effectiveness in viral eradication and tolerability, DAA agents can be used in all HCV patients including those with advanced hepatic impairment. The optimal timing of HCV therapy in LT candidates is long debated. DAA agents have shown to improve the hepatic function to the degree where transplantation may no longer be required in some LT candidates whereas in others, the reduction in MELD score may not necessarily improve poor quality of life. Therefore, it is crucial to identify such candidates to maintain the access to LT and treat HCV in post-transplant setting. The viral eradication and improvement in hepatic function with subsequent delisting from LT waitlist is not only cost-effective but has a substantial societal benefit in the setting of limited donor pool. The available data suggests that LT candidates with mild to moderate impairment benefit from pre-transplant HCV treatment, whereas post-transplant DAA therapy may be employed for those in whom LT cannot be avoided. However, the decision to treat LT candidates should also reflect patient preferences, local/regional waitlist parameters and specific needs of a transplant center. Nevertheless, future studies are needed to identify the accurate predictors of improvement in quality of life and hepatic function in LT candidates following DAA therapy to better guide the providers caring for these patients.

## REFERENCES

- 1 **Denniston MM**, Jiles RB, Drobeniuc J, Kleven RM, Ward JW, McQuillan GM, Holmberg SD. Chronic hepatitis C virus infection in the United States, National Health and Nutrition Examination Survey 2003 to 2010. *Ann Intern Med* 2014; **160**: 293-300 [PMID: 24737271 DOI: 10.7326/m13-1133]
- 2 **Edlin BR**, Eckhardt BJ, Shu MA, Holmberg SD, Swan T. Toward a more accurate estimate of the prevalence of hepatitis C in the United States. *Hepatology* 2015; **62**: 1353-1363 [PMID: 26171595 DOI: 10.1002/hep.27978]
- 3 **Ryerson AB**, Ehemann CR, Altekruse SF, Ward JW, Jemal A, Sherman RL, Henley SJ, Holtzman D, Lake A, Noone AM, Anderson RN, Ma J, Ly KN, Cronin KA, Penberthy L, Kohler BA. Annual Report to the Nation on the Status of Cancer, 1975-2012, featuring the increasing incidence of liver cancer. *Cancer* 2016; **122**: 1312-1337 [PMID: 26959385 DOI: 10.1002/cncr.29936]
- 4 **Crespo G**, Mariño Z, Navasa M, Forns X. Viral hepatitis in liver transplantation. *Gastroenterology* 2012; **142**: 1373-1383.e1 [PMID: 22537446 DOI: 10.1053/j.gastro.2012.02.011]
- 5 **Moyer VA**; U.S. Preventive Services Task Force. Screening for hepatitis C virus infection in adults: U.S. Preventive Services Task Force recommendation statement. *Ann Intern Med* 2013; **159**: 349-357 [PMID: 23798026 DOI: 10.7326/0003-4819-159-5-201309030-00672]
- 6 **Chhatwal J**, Wang X, Ayer T, Kabiri M, Chung RT, Hur C, Donohue JM, Roberts MS, Kanwal F. Hepatitis C Disease Burden in the United States in the era of oral direct-acting antivirals. *Hepatology* 2016; **64**: 1442-1450 [PMID: 27015107 DOI: 10.1002/hep.28571]
- 7 **van der Meer AJ**, Veldt BJ, Feld JJ, Wedemeyer H, Dufour JF, Lammert F, Duarte-Rojo A, Heathcote EJ, Manns MP, Kuske L, Zeuzem S, Hofmann WP, de Knecht RJ, Hansen BE, Janssen HL. Association between sustained virological response and all-cause mortality among patients with chronic hepatitis C and advanced hepatic fibrosis. *JAMA* 2012; **308**: 2584-2593 [PMID: 23268517 DOI: 10.1001/jama.2012.144878]
- 8 **Bunchorntavakul C**, Reddy KR. Treat chronic hepatitis C virus infection in decompensated cirrhosis - pre- or post-liver transplantation? the ironic conundrum in the era of effective and well-tolerated therapy. *J Viral Hepat* 2016; **23**: 408-418 [PMID: 27018088 DOI: 10.1111/jvh.12534]
- 9 **Carrion AF**, Khaderi SA, Sussman NL. Model for end-stage liver disease limbo, model for end-stage liver disease purgatory, and the dilemma of treating hepatitis C in patients awaiting liver transplantation. *Liver Transpl* 2016; **22**: 279-280 [PMID: 26663608 DOI: 10.1002/lt.24383]
- 10 **Lingala S**, Ghany MG. Natural History of Hepatitis C. *Gastroenterol Clin North Am* 2015; **44**: 717-734 [PMID: 26600216 DOI: 10.1016/j.gtc.2015.07.003]
- 11 **Davis GL**, Alter MJ, El-Serag H, Poynard T, Jennings LW. Aging of hepatitis C virus (HCV)-infected persons in the United States: a multiple cohort model of HCV prevalence and disease progression. *Gastroenterology* 2010; **138**: 513-521, 521.e1-521.e6 [PMID: 19861128 DOI: 10.1053/j.gastro.2009.09.067]
- 12 **Wray CM**, Davis AM. Screening for hepatitis C. *JAMA* 2015; **313**: 1855-1856 [PMID: 25965235 DOI: 10.1001/jama.2015.2833]
- 13 **Snowberger N**, Chinnakotla S, Lepe RM, Peattie J, Goldstein R, Klintmalm GB, Davis GL. Alpha fetoprotein, ultrasound, computerized tomography and magnetic resonance imaging for detection of hepatocellular carcinoma in patients with advanced cirrhosis. *Aliment Pharmacol Ther* 2007; **26**: 1187-1194 [PMID: 17944733 DOI: 10.1111/j.1365-2036.2007.03498.x]
- 14 **Bunchorntavakul C**, Reddy KR. Management of Hepatitis C Before and After Liver Transplantation in the Era of Rapidly Evolving Therapeutic Advances. *J Clin Transl Hepatol* 2014; **2**: 124-133 [PMID: 26357623 DOI: 10.14218/JCTH.2014.00002]
- 15 **Ferrarese A**, Zanetto A, Gambato M, Bortoluzzi I, Nadal E, Germani G, Senzolo M, Burra P, Russo FP. Liver transplantation for viral hepatitis in 2015. *World J Gastroenterol* 2016; **22**: 1570-1581 [PMID: 26819523 DOI: 10.3748/wjg.v22.i4.1570]
- 16 **Kogiso T**, Hashimoto E, Ikarashi Y, Kodama K, Taniai M, Torii N, Egawa H, Yamamoto M, Tokushige K. Spontaneous clearance of HCV accompanying hepatitis after liver transplantation. *Clin J Gastroenterol* 2015; **8**: 323-329 [PMID: 26342292 DOI: 10.1007/s12328-015-0602-y]
- 17 **Berenguer M**, López-Labrador FX, Wright TL. Hepatitis C and liver transplantation. *J Hepatol* 2001; **35**: 666-678 [PMID: 11690716]
- 18 **Berenguer M**, Rayón JM, Prieto M, Aguilera V, Nicolás D, Ortiz V, Carrasco D, López-Andujar R, Mir J, Berenguer J. Are posttransplantation protocol liver biopsies useful in the long term? *Liver Transpl* 2001; **7**: 790-796 [PMID: 11552213 DOI: 10.1053/jlts.2001.23794]
- 19 **Berenguer M**, Prieto M, Rayón JM, Mora J, Pastor M, Ortiz V, Carrasco D, San Juan F, Burgueño MD, Mir J, Berenguer J. Natural history of clinically compensated hepatitis C virus-related graft cirrhosis after liver transplantation. *Hepatology* 2000; **32**: 852-858 [PMID: 11003634 DOI: 10.1053/jhep.2000.17924]
- 20 **Pruthi J**, Medkiff KA, Esrason KT, Donovan JA, Yoshida EM, Erb SR, Steinbrecher UP, Fong TL. Analysis of causes of death in liver transplant recipients who survived more than 3 years. *Liver Transpl* 2001; **7**: 811-815 [PMID: 11552217 DOI: 10.1053/jlts.2001.27084]
- 21 **Fattovich G**, Giustina G, Degos F, Tremolada F, Diodati G, Almasio P, Nevens F, Solinas A, Mura D, Brouwer JT, Thomas

- H, Njapoum C, Casarin C, Bonetti P, Fuschi P, Basho J, Tocco A, Bhalla A, Galassini R, Noventa F, Schalm SW, Realdi G. Morbidity and mortality in compensated cirrhosis type C: a retrospective follow-up study of 384 patients. *Gastroenterology* 1997; **112**: 463-472 [PMID: 9024300 DOI: 10.1053/gast.1997.v112.pm9024300]
- 22 **Niederer C**, Lange S, Heintges T, Erhardt A, Buschkamp M, Hürter D, Nawrocki M, Kruska L, Hensel F, Petry W, Häussinger D. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. *Hepatology* 1998; **28**: 1687-1695 [PMID: 9828236 DOI: 10.1002/hep.510280632]
  - 23 **Forman LM**, Lewis JD, Berlin JA, Feldman HI, Lucey MR. The association between hepatitis C infection and survival after orthotopic liver transplantation. *Gastroenterology* 2002; **122**: 889-896 [PMID: 11910340 DOI: 10.1053/gast.2002.32418]
  - 24 **Curry MP**, Forns X, Chung RT, Terrault NA, Brown R Jr, Fenkel JM, Gordon F, O'Leary J, Kuo A, Schiano T, Everson G, Schiff E, Befeler A, Gane E, Saab S, McHutchison JG, Subramanian GM, Symonds WT, Denning J, McNair L, Arterburn S, Svarovskaia E, Moonka D, Afdhal N. Sofosbuvir and ribavirin prevent recurrence of HCV infection after liver transplantation: an open-label study. *Gastroenterology* 2015; **148**: 100-107.e1 [PMID: 25261839 DOI: 10.1053/j.gastro.2014.09.023]
  - 25 **Charlton M**, Everson GT, Flamm SL, Kumar P, Landis C, Brown RS Jr, Fried MW, Terrault NA, O'Leary JG, Vargas HE, Kuo A, Schiff E, Sulkowski MS, Gilroy R, Watt KD, Brown K, Kwo P, Pungpapong S, Korenblat KM, Muir AJ, Teperman L, Fontana RJ, Denning J, Arterburn S, Dvory-Sobol H, Brandt-Sarif T, Pang PS, McHutchison JG, Reddy KR, Afdhal N; SOLAR-1 Investigators. Ledipasvir and Sofosbuvir Plus Ribavirin for Treatment of HCV Infection in Patients With Advanced Liver Disease. *Gastroenterology* 2015; **149**: 649-659 [PMID: 25985734 DOI: 10.1053/j.gastro.2015.05.010]
  - 26 **Tada T**, Kumada T, Toyoda H, Kiriya S, Tanikawa M, Hisanaga Y, Kanamori A, Kitabatake S, Yama T, Tanaka J. Viral eradication reduces all-cause mortality, including non-liver-related disease, in patients with progressive hepatitis C virus-related fibrosis. *J Gastroenterol Hepatol* 2017; **32**: 687-694 [PMID: 27577675 DOI: 10.1111/jgh.13589]
  - 27 **Everson GT**, Terrault NA, Lok AS, Rodrigo del R, Brown RS Jr, Saab S, Shiffman ML, Al-Osaimi AM, Kulik LM, Gillespie BW, Everhart JE; Adult-to-Adult Living Donor Liver Transplantation Cohort Study. A randomized controlled trial of pretransplant antiviral therapy to prevent recurrence of hepatitis C after liver transplantation. *Hepatology* 2013; **57**: 1752-1762 [PMID: 22821361 DOI: 10.1002/hep.25976]
  - 28 **Osinusi A**, Meissner EG, Lee YJ, Bon D, Heytens L, Nelson A, Sneller M, Kohli A, Barrett L, Proschan M, Herrmann E, Shivakumar B, Gu W, Kwan R, Teferi G, Talwani R, Silk R, Kotb C, Wroblewski S, Fishbein D, Dewar R, Highbarger H, Zhang X, Kleiner D, Wood BJ, Chavez J, Symonds WT, Subramanian M, McHutchison J, Polis MA, Fauci AS, Masur H, Kottlil S. Sofosbuvir and ribavirin for hepatitis C genotype 1 in patients with unfavorable treatment characteristics: a randomized clinical trial. *JAMA* 2013; **310**: 804-811 [PMID: 23982366 DOI: 10.1001/jama.2013.109309]
  - 29 **Feld JJ**, Jacobson IM, Hézode C, Asselah T, Ruane PJ, Gruener N, Abergel A, Mangia A, Lai CL, Chan HL, Mazzotta F, Moreno C, Yoshida E, Shafraan SD, Towner WJ, Tran TT, McNally J, Osinusi A, Svarovskaia E, Zhu Y, Brainard DM, McHutchison JG, Agarwal K, Zeuzem S; ASTRAL-1 Investigators. Sofosbuvir and Velpatasvir for HCV Genotype 1, 2, 4, 5, and 6 Infection. *N Engl J Med* 2015; **373**: 2599-2607 [PMID: 26571066 DOI: 10.1056/NEJMoa1512610]
  - 30 **Foster GR**, Afdhal N, Roberts SK, Bräu N, Gane EJ, Pianko S, Lawitz E, Thompson A, Shiffman ML, Cooper C, Towner WJ, Conway B, Ruane P, Bourlière M, Asselah T, Berg T, Zeuzem S, Rosenberg W, Agarwal K, Stedman CA, Mo H, Dvory-Sobol H, Han L, Wang J, McNally J, Osinusi A, Brainard DM, McHutchison JG, Mazzotta F, Tran TT, Gordon SC, Patel K, Reau N, Mangia A, Sulkowski M; ASTRAL-2 Investigators; ASTRAL-3 Investigators. Sofosbuvir and Velpatasvir for HCV Genotype 2 and 3 Infection. *N Engl J Med* 2015; **373**: 2608-2617 [PMID: 26575258 DOI: 10.1056/NEJMoa1512612]
  - 31 **Saab S**, Park SH, Mizokami M, Omata M, Mangia A, Eggleston E, Zhu Y, Knox SJ, Pang P, Subramanian M, Kowdley K, Afdhal NH. Safety and efficacy of ledipasvir/sofosbuvir for the treatment of genotype 1 hepatitis C in subjects aged 65 years or older. *Hepatology* 2016; **63**: 1112-1119 [PMID: 26704693 DOI: 10.1002/hep.28425]
  - 32 **Foster GR**, Irving WL, Cheung MC, Walker AJ, Hudson BE, Verma S, McLauchlan J, Mutimer DJ, Brown A, Gelson WT, MacDonald DC, Agarwal K; HCV Research, UK. Impact of direct acting antiviral therapy in patients with chronic hepatitis C and decompensated cirrhosis. *J Hepatol* 2016; **64**: 1224-1231 [PMID: 26829205 DOI: 10.1016/j.jhep.2016.01.029]
  - 33 **Samuel D**, Manns M, Forns X, Flamm SL, Reddy KR, Denning J, Arterburn S, Brandt-Sarif T, Pang PS, McHutchison JG, Afdhal N, Charlton M, Gane E, Mutimer D, Everson GT. P0774: Ledipasvir/sofosbuvir with ribavirin is safe in >600 decompensated and post liver transplantation patients with HCV infection: An integrated safety analysis of the solar 1 and solar 2 trials. *J Hepatol* 2015; **62**: S620-S621 [DOI: 10.1016/S0168-8278(15)30977-6]
  - 34 **Goldberg DS**, Blumberg E, McCauley M, Abt P, Levine M. Improving Organ Utilization to Help Overcome the Tragedies of the Opioid Epidemic. *Am J Transplant* 2016; **16**: 2836-2841 [PMID: 27438538 DOI: 10.1111/ajt.13971]
  - 35 **Tapper EB**, Afdhal NH, Curry MP. Before or After Transplantation? A Review of the Cost Effectiveness of Treating Waitlisted Patients With Hepatitis C. *Transplantation* 2017; **101**: 933-937 [PMID: 28437385 DOI: 10.1097/tp.0000000000001611]
  - 36 **Younossi ZM**, Park H, Saab S, Ahmed A, Dieterich D, Gordon SC. Cost-effectiveness of all-oral ledipasvir/sofosbuvir regimens in patients with chronic hepatitis C virus genotype 1 infection. *Aliment Pharmacol Ther* 2015; **41**: 544-563 [PMID: 25619871 DOI: 10.1111/apt.13081]
  - 37 **Belli LS**, Berenguer M, Cortesi PA, Strazzabosco M, Rockenschaub SR, Martini S, Morelli C, Donato F, Volpes R, Pageaux GP, Coilly A, Fagioli S, Amadeo G, Perricone G, Vinaixa C, Berlakovich G, Facchetti R, Polak W, Muesan P, Duvoux C; European Liver and Intestine Association (ELITA). Delisting of liver transplant candidates with chronic hepatitis C after viral eradication: A European study. *J Hepatol* 2016; **65**: 524-531 [PMID: 27212241 DOI: 10.1016/j.jhep.2016.05.010]
  - 38 **Picciotto FP**, Tritto G, Lanza AG, Addario L, De Luca M, Di Costanzo GG, Lampasi F, Tartaglione MT, Marsilia GM, Calise F, Cuomo O, Ascione A. Sustained virological response to antiviral therapy reduces mortality in HCV reinfection after liver transplantation. *J Hepatol* 2007; **46**: 459-465 [PMID: 17196700 DOI: 10.1016/j.jhep.2006.10.017]
  - 39 **Berenguer M**. Systematic review of the treatment of established recurrent hepatitis C with pegylated interferon in combination with ribavirin. *J Hepatol* 2008; **49**: 274-287 [PMID: 18571272 DOI: 10.1016/j.jhep.2008.05.002]
  - 40 **Charlton M**, Gane E, Manns MP, Brown RS Jr, Curry MP, Kwo PY, Fontana RJ, Gilroy R, Teperman L, Muir AJ, McHutchison JG, Symonds WT, Brainard D, Kirby B, Dvory-Sobol H, Denning J, Arterburn S, Samuel D, Forns X, Terrault NA. Sofosbuvir and ribavirin for treatment of compensated recurrent hepatitis C virus infection after liver transplantation. *Gastroenterology* 2015; **148**: 108-117 [PMID: 25304641 DOI: 10.1053/j.gastro.2014.10.001]
  - 41 **Crittenden NE**, Buchanan LA, Pinkston CM, Cave B, Barve A, Marsano L, McClain CJ, Jones CM, Marvin MR, Davis EG, Kuns-Adkins CB, Gedaly R, Brock G, Shah MB, Rosenau J, Cave MC. Simeprevir and sofosbuvir with or without ribavirin to treat recurrent genotype 1 hepatitis C virus infection after orthotopic liver transplantation. *Liver Transpl* 2016; **22**: 635-643 [PMID: 26915588 DOI: 10.1002/lt.24422]
  - 42 **Jackson WE**, Hanouneh M, Apfel T, Alkhouri N, John BV, Zervos X, Zein NN, Hanouneh IA. Sofosbuvir and simeprevir

- without ribavirin effectively treat hepatitis C virus genotype 1 infection after liver transplantation in a two-center experience. *Clin Transplant* 2016; **30**: 709-713 [PMID: 27019204 DOI: 10.1111/ctr.12738]
- 43 **Poordad F**, Schiff ER, Vierling JM, Landis C, Fontana RJ, Yang R, McPhee F, Hughes EA, Noviello S, Swenson ES. Daclatasvir with sofosbuvir and ribavirin for hepatitis C virus infection with advanced cirrhosis or post-liver transplantation recurrence. *Hepatology* 2016; **63**: 1493-1505 [PMID: 26754432 DOI: 10.1002/hep.28446]
  - 44 **AASLD/IDSA HCV Guidance Panel**. Hepatitis C guidance: AASLD-IDSA recommendations for testing, managing, and treating adults infected with hepatitis C virus. *Hepatology* 2015; **62**: 932-954 [PMID: 26111063 DOI: 10.1002/hep.27950]
  - 45 **Forns X**, Charlton M, Denning J, McHutchison JG, Symonds WT, Brainard D, Brandt-Sarif T, Chang P, Kivett V, Castells L, Prieto M, Fontana RJ, Baumert TF, Coilly A, Londoño MC, Habersetzer F. Sofosbuvir compassionate use program for patients with severe recurrent hepatitis C after liver transplantation. *Hepatology* 2015; **61**: 1485-1494 [PMID: 25557906 DOI: 10.1002/hep.27681]
  - 46 **Pascasio JM**, Vinaixa C, Ferrer MT, Colmenero J, Rubin A, Castells L, Manzano ML, Lorente S, Testillano M, Xiol X, Molina E, González-Diéguez L, Otón E, Pascual S, Santos B, Herrero JI, Salcedo M, Montero JL, Sánchez-Antolín G, Narváez I, Nogueras F, Giraldez Á, Prieto M, Forns X, Londoño MC. Clinical outcomes of patients undergoing antiviral therapy while awaiting liver transplantation. *J Hepatol* 2017; **67**: 1168-1176 [PMID: 28842296 DOI: 10.1016/j.jhep.2017.08.008]
  - 47 **Verna EC**. The dynamic landscape of liver transplant in the era of effective hepatitis C virus therapy. *Hepatology* 2017; **65**: 763-766 [PMID: 28093781 DOI: 10.1002/hep.29054]
  - 48 **Aggarwal A**, Yoo ER, Perumpail RB, Cholankeril G, Kumari R, Daugherty TJ, Lapasaran AS, Ahmed A. Sofosbuvir Use in the Setting of End-stage Renal Disease: A Single Center Experience. *J Clin Transl Hepatol* 2017; **5**: 23-26 [PMID: 28507922 DOI: 10.14218/jcth.2016.00060]
  - 49 **Nazario HE**, Ndungu M, Modi AA. Sofosbuvir and simeprevir in hepatitis C genotype 1-patients with end-stage renal disease on haemodialysis or GFR <30 ml/min. *Liver Int* 2016; **36**: 798-801 [PMID: 26583882 DOI: 10.1111/liv.13025]

**P-Reviewer:** Kakaei F, Marzaban R **S-Editor:** Gong ZM  
**L-Editor:** A **E-Editor:** Li RF





## Basic Study

# circRNA\_0046366 inhibits hepatocellular steatosis by normalization of PPAR signaling

Xing-Ya Guo, Fang Sun, Jian-Neng Chen, Yu-Qin Wang, Qin Pan, Jian-Gao Fan

Xing-Ya Guo, Fang Sun, Yu-Qin Wang, Qin Pan, Jian-Gao Fan, Department of Gastroenterology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China

Jian-Neng Chen, Department of Hepatology, Zhengxing Hospital, Zhangzhou 363000, Fujian Province, China

Jian-Gao Fan, Shanghai Key Laboratory of Children's Digestion and Nutrition, Shanghai 200092, China

ORCID number: Xing-Ya Guo (0000-0002-2644-5126); Fang Sun (0000-0002-6688-4676); Jian-Neng Chen (0000-0002-0728-0813); Yu-Qin Wang (0000-0003-2894-6220); Qin Pan (0000-0001-5855-4952); Jian-Gao Fan (0000-0001-7443-5056).

**Author contributions:** Pan Q and Fan JG should be as the co-corresponding authors; Guo XY, Sun F and Chen JN contributed equally to this paper; Pan Q and Fan JG conceived and designed the experiments; Guo XY and Sun F performed the experiments; Chen JN, Wang YQ and Pan Q analyzed the data; Pan Q wrote the paper.

**Supported by** National Key Research and Development Plan 'Precision Medicine Research', No. 2017YFSF090203; National Natural Science Foundation of China, No. 81070346, No. 81270492, No. 81470859, No. 81270491 and No. 81470840; State Key Development Program for Basic Research of China, No. 2012CB517501; 100 Talents Program, No. XBR2011007h; and Program of the Committee of Science and Technology, No. 09140903500.

**Institutional review board statement:** This paper was approved by the Xinhua Hospital Ethics Committee Affiliated to Shanghai Jiaotong University School of Medicine.

**Conflict-of-interest statement:** No conflict of interest is declared for each author of the manuscript.

**Data sharing statement:** Technical appendix, statistical code, and dataset available from the authors at [fanjiangao@xinhumed.com.cn](mailto:fanjiangao@xinhumed.com.cn) or [panqin@xinhumed.com.cn](mailto:panqin@xinhumed.com.cn). Participants gave informed consent for data sharing.

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**Manuscript source:** Unsolicited manuscript

**Correspondence to:** Qin Pan, MD, PhD, Professor, Department of Gastroenterology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Kongjiang Road NO 1665, Yangpu District, Shanghai 200092, China. [panqin@xinhumed.com.cn](mailto:panqin@xinhumed.com.cn)  
**Telephone:** +86-21-25078999  
**Fax:** +86-21-25077340

**Received:** October 7, 2017

**Peer-review started:** October 9, 2017

**First decision:** October 25, 2017

**Revised:** November 15, 2017

**Accepted:** November 27, 2017

**Article in press:** November 27, 2017

**Published online:** January 21, 2018

## Abstract

### AIM

To investigate micro (mi)R-34a-antagonizing circular (circ)RNA that underlies hepatocellular steatosis.

### METHODS

The effect of circRNA on miR-34a was recognized by the miRNA response element (MRE), and validated by the dual-luciferase reporter assay. Its association with hepatocellular steatosis was investigated in HepG2-based hepatocellular steatosis induced by free fatty acids (FFAs; 2:1 oleate:palmitate) stimulation. After normalization of the steatosis-related circRNA by expression vector, analysis of miR-34a activity,

peroxisome proliferator-activated receptor (PPAR) $\alpha$  level, and expression of downstream genes were carried out so as to reveal its impact on the miR-34a/PPAR $\alpha$  regulatory system. Both triglyceride (TG) assessment and cytopathological manifestations uncovered the role of circRNA in miR-34a-dependent hepatosteatogenesis.

## RESULTS

Bioinformatic and functional analysis verified circRNA\_0046366 to antagonize the activity of miR-34a *via* MRE-based complementation. In contrast to its lowered level during FFA-induced hepatocellular steatosis, circRNA\_0046366 up-regulation abolished the miR-34a-dependent inhibition of PPAR $\alpha$  that played a critical role in metabolic signaling pathways. PPAR $\alpha$  restoration exerted transcriptional improvement to multiple genes responsible for lipid metabolism. TG-specific lipolytic genes [carnitine palmitoyltransferase 1A (CPT1A) and solute-carrier family 27A (SLC27A)] among these showed significant increase in their expression levels. The circRNA\_0046366-related rebalancing of lipid homeostasis led to dramatic reduction of TG content, and resulted in the ameliorated phenotype of hepatocellular steatosis.

## CONCLUSION

Dysregulation of circRNA\_0046366/miR-34a/PPAR $\alpha$  signaling may be a novel epigenetic mechanism underlying hepatocellular steatosis. circRNA\_0046366 serves as a potential target for the treatment of hepatic steatosis.

**Key words:** Hepatocytes; Steatosis; circRNA\_0046366; miR-34a; Peroxisome proliferator-activated receptor  $\alpha$

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**Core tip:** circRNA\_0046366, which demonstrated expression loss in HepG2-based hepatocellular steatosis, exerts antagonistic effect on miR-34a activity. miR-34a inactivation abrogates its inhibitory role against peroxisome proliferator-activated receptor (PPAR) $\alpha$ , and then rescues the PPAR $\alpha$  level. PPAR $\alpha$  restoration further improves the expression of downstream genes [*i.e.* carnitine palmitoyltransferase 1A (CPT1A) and solute-carrier family 27A (SLC27A)], at both transcriptional and translational levels, which are associated to triglyceride metabolism. In conclusion, the rebalancing of lipid homeostasis down-regulates triglyceride content, and attenuates the hepatocellular steatosis.

Guo XY, Sun F, Chen JN, Wang YQ, Pan Q, Fan JG. circRNA\_0046366 inhibits hepatocellular steatosis by normalization of PPAR signaling. *World J Gastroenterol* 2018; 24(3): 323-337 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i3/323.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i3.323>

## INTRODUCTION

Hepatic steatosis is associated with hepatocyte-specific accumulation of lipid droplets ( $\geq 5\%$  of volume or weight), and has increased dramatically worldwide with the growing incidence of obesity<sup>[1,2]</sup>. Hepatic steatosis is one of the most common chronic liver diseases in western countries and the Asia-Pacific area<sup>[2-6]</sup>. It is a critical step in the development of nonalcoholic fatty liver disease, which ranges from simple steatosis to nonalcoholic steatohepatitis, liver fibrosis/cirrhosis, and finally hepatocellular carcinoma<sup>[7,8]</sup>. Patients with hepatic steatosis are also susceptible to some aspects of metabolic syndrome (*e.g.*, type 2 diabetes and hyperlipidemia)<sup>[9,10]</sup> and related diseases (*i.e.* cardiovascular events, cerebrovascular diseases, and extrahepatic cancer)<sup>[11,12]</sup>. Although it is being recognized as a public health burden, clinical intervention for hepatic steatosis is deficient because of the limited understanding of its underlying mechanisms.

Micro (mi)RNAs have been identified as critical regulators of various physiological processes and diseases<sup>[13,14]</sup>. miR-34a is significantly up-regulated in different models of rodent hepatic steatosis<sup>[15,16]</sup>. Consistently, miR-34a expression correlates well with the clinical occurrence of hepatic steatosis in the Chinese, Japanese and Philippine populations<sup>[17-19]</sup>. The increased level of circulating miR-34a discriminates patients with hepatic steatosis from healthy controls with area under the curve (AUC) of 0.781<sup>[19]</sup>. Targeted repression of peroxisome proliferator-activated receptor (PPAR) $\alpha$ , which maintains homeostasis of lipid metabolism in hepatocytes, highlights the steatosis-related effect of miR-34a<sup>[20]</sup>. Antagonism of miR-34a, therefore, has potential in the therapy of hepatic steatosis.

Circular (circ)RNAs are a novel class of non-coding RNAs containing miRNA response elements (MREs)<sup>[21]</sup> that can be used to investigate miRNA-specific antagonists. circRNA\_000203 in angiotensin-II-induced cardiac fibroblasts inactivates miR-26b-5p by the complementation between MREs and miRNAs<sup>[22]</sup>. circRNA\_010567 in diabetic mice abrogates the effect of miR-141 in a same manner<sup>[23]</sup>. The miRNA-dependent inhibition of target mRNAs (transforming growth factor- $\beta$ 1, collagen 1a2 and connective tissue growth factor) is then abolished, which results in myocardial fibrosis<sup>[22,23]</sup>. By serving as a natural "miR-223 sponge", heart-related circRNA (HRCR) sequesters and antagonizes its inhibitory role against activity-regulated cytoskeleton-associated protein (ARC)<sup>[24]</sup>. Thus, pathological hypertrophic responses and heart failure are prevented by the HRCR/miR-223/ARC axis<sup>[24]</sup>. However, both miR-34a-targeting circRNA and its actions are rarely reported in hepatic steatosis.

Employing databases of non-coding RNA (circBase and miRBase) and algorithms of circRNA-miRNA

Table 1 Primers for real-time PCR

Gene	Primer sequence, 5'-3'		Product, nt
hsa_circ_000366	F: CGTCCATTCGTTTGTGAGCC	R: CTTACAGCCTCATCGGAGC	126
APOA1	F: GCCAGGCTCGGCATTCTG	R: GCCGCTGTCTTTGAGCACATCCA	104
APOC3	F: CGGGTACTCCTTGTGTGTC	R: TTGTCTTAACGGTGCTCCA	230
CPT1A	F: CCAGACGAAGAACGTGGTCA	R: ATCTTGCCGTGCTCAGTGAA	132
FASN	F: ATGAGCACCAACGACACGAT	R: CTATAGGCCGAGCCTTCTC	140
HMGCS	F: TGGTTCCCTTGCATCIGTTC	R: TTATCAAGAGCAGACCCCGG	150
LPL	F: CATTCCCGGAGTAGCAGAGT	R: GGACACTGGGTAATGCTCCT	205
PPAR $\alpha$	F: CCCCTCCTCGGTGACTTATC	R: ATTCGTCCAAAACGAATCGCGT	297
SLC27A	F: GGCCCAACGACATCGTCTAT	R: TAGCGGCACAGTTCACCAAT	190
VLDLR	F: GTAGGCAAAGAGCCAAGTC	R: GTACACCCAATCAACAGCA	264
U6	F: ATTGAACGATACAGAGAAGATT	R: GGAACGCTTCACGAATTIG	70
GAPDH	F: CTGAACGGGAAGCTCACTGG	R: AAAGTGGTCGTTGAGGGCAA	252

interaction, circRNA\_0046366 is now recognized to be a specific antagonist of miR-34a. To reveal the circRNA-miRNA interaction underlying hepatocellular steatosis, expression patterns of circRNA\_0046366 and miR-34a were investigated in a model of HepG2-based steatosis model in the presence of high-fat stimulation. The antagonistic effect of circRNA\_0046366 on miR-34a was evaluated by bioinformatic analysis and dual-luciferase reporter assay. After the up-regulation of hepatocellular circRNA\_0046366, functional experiments exhibited its impact on miR-34a, key miRNA-target (PPAR $\alpha$ ), and downstream genes responsible for lipid metabolism. Phenotypic identification finally revealed the pathophysiological role of circRNA\_0046366/miR-34a/PPAR $\alpha$  signaling in hepatocellular steatogenesis.

## MATERIALS AND METHODS

### Establishment of hepatocellular steatosis by high-fat stimulation

Exponentially growing HepG2 cells (Cell Bank of Type Culture Collection, Shanghai, China) were seeded in a 6-well plate at  $2 \times 10^5$ /well, and randomized into groups of normal and steatosis (9 wells/group). In contrast to those cultured in Dulbecco's modified Eagle's medium, penicillin-streptomycin, and 10% fetal bovine serum (control group), HepG2 cells in the model group were subjected to additional exposure to 0.5 mmol/L FFA (oleate:palmitate = 2:1; Sigma-Aldrich, St. Louis, MO, United States) for 24 h<sup>[25]</sup>.

### Assessment of circRNA\_0046366 expression

Total RNA of each sample was extracted by the phenol/chloroform method, and then treated by the ExScript RT Reagent Kit (TaKaRa, Kusatsu, Japan) for reverse transcription (RT). Quantitative real-time polymerase chain reaction (PCR) was performed, with primers specific to cDNA of circRNA\_0046366 (Table 1)<sup>[24]</sup>, using SYBR Premix ExTaq (TaKaRa) on the Applied Biosystems 7500 Real-Time PCR Detection Systems (Bio-Rad Laboratories, Hercules, CA, United States).

The expression level of hepatocellular circRNA\_0046366 was evaluated against U6 by the  $2^{-\Delta\Delta Ct}$  method.

### Bioinformatic analysis

The effect of circRNA\_0046366 on miRNAs was analyzed by the CircInteractome method according to MRE-based circRNA/miRNA complementation<sup>[26]</sup>, and then the set of circRNA\_0046366-targeting miRNAs was constructed. On the other side, the experiment-proven, hepatic steatosis-inducing miRNAs were pooled to establish another miRNA set<sup>[27]</sup>. Key target miRNAs that play an essential part in circRNA-related hepatocellular steatosis were successively filtered by the intersection of circRNA\_0046366-targeting miRNA set and hepatic steatosis-inducing set.

To shed light on their actions associated with hepatocellular steatosis, a miRNA-specific targetome was constructed using genes obtained from miRBase<sup>[28]</sup>. According to the annotations of Kyoto Encyclopedia of Genes and Genomes (KEGG) database<sup>[29]</sup>, enrichment scoring, Fisher's exact test and false discovery rate (FDR) analysis were further employed to recognize the miRNA-regulating signal pathways<sup>[30]</sup>. Finally, gene interaction was illustrated within the top-enrichment pathways using databases of KEGG and PubMed<sup>[31]</sup>.

### Luciferase reporter assays

hsa-circRNA\_0046366 (CircBase; Rajewsky Laboratory, Berlin, Germany) with putative target sites for miR-34a was synthesized and cloned into the pMIR-REPORT vector (Thermo Fisher Scientific, Waltham, MA, United States) for the purpose of constructing recombinant reporter vector (pMIR-REPORT-circRNA\_0046366-wildtype)<sup>[32]</sup>. Reporter vector with mutant circRNA\_0046366 (pMIR-REPORT-circRNA\_0046366-mutant) was also generated by deletion of an MRE sequence. As defined by the relative activity of firefly luciferase against Renilla luciferase, cotransfection of reporter vector (pMIR-REPORT-circRNA\_0046366-wildtype or pMIR-REPORT-circRNA\_0046366-mutant) and oligonucleotides (miR-34a mimics or negative control) revealed circRNA-miRNA interaction using a dual-luciferase assay kit

(Promega, Madison, WI, United States).

### **circRNA administration**

cDNA of circRNA\_0046366 was synthesized according to the sequence obtained from circBase database (www.circbase.org), and then cloned into H3790 pcDNA3.1 plasmid using TA Cloning™ Kit (Thermo Fisher Scientific) as per the manufacturer's instructions<sup>[33]</sup>. DNA sequencing verified the construction of recombinant vector (pcDNA3.1(+)-GFP-circRNA\_0046366) expressing circRNA\_0046366.

HepG2 cells in the exponential phase were randomly divided into 6 groups, including normal, steatosis, control, circRNA, circRNA+mimics, and circRNA+mimics normal control (NC) group ( $2 \times 10^5$  cells/well, 9 wells/group). In contrast to those without circRNA\_0046366 regulation (normal and steatosis groups), the circRNA, circRNA+mimics, and circRNA+mimics NC groups were exposed to 12-h transfection of pcDNA3.1(+)-GFP-circRNA\_0046366 (4 µg/well), pcDNA3.1(+)-GFP-circRNA\_0046366 (4 µg/well) + miR-34a mimics (Genechem, Shanghai, China; 50 pmol/well), and pcDNA3.1(+)-GFP-circRNA\_0046366 (4 µg/well) + miR-34a mimics, negative control (50 pmol/well; Genechem, Shanghai, China), respectively, using Lipofectamine 2000<sup>[34]</sup>. Transfection of blank plasmid (pcDNA3.1(+)-GFP at 4 µg/well) was applied to the control group. All groups, with the exception of the normal group, received FFA treatment for a further 24 h as mentioned above. After the transfection of circRNA-containing plasmid, the expression level of circRNA\_0046366 was dynamically assessed in the circRNA group at the time points of 24, 48, and 72 h.

### **Real-time RT-PCR**

cDNA of miR-34a was generated by the Mir-X miRNA First Strand Synthesis Kit (TaKaRa) using total RNA samples from each group. After normalization against an internal control (U6), real-time PCR demonstrated expression of hepatocellular miR-34a by the SYBR Fast qPCR Mix (TaKaRa)<sup>[20]</sup>. RT and real-time PCR for PPAR $\alpha$ , apolipoprotein A1 (APOA1), apolipoprotein C3 (APOC3), fatty acid synthase (FASN), lipoprotein lipase (LPL), 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS), very low-density lipoprotein receptor (VLDLR), carnitine palmitoyltransferase 1A (CPT1A), solute-carrier family 27A (SLC27A), and GAPDH were carried out using the ExScript RT Reagent Kit (TaKaRa), and SYBR Premix ExTaq (TaKaRa), respectively, using standard procedures. Gene-specific primers of these reactions were designed by Premier 5.0 software (PREMIER Biosoft, Palo Alto, CA, United States) (Table 1). According to the results obtained from the Applied Biosystems 7500 Real-Time PCR Detection Systems (Bio-Rad Laboratories), expression of these genes was assessed based on the  $2^{-\Delta\Delta Ct}$  method.

### **Western blotting**

Total protein of each sample was prepared using

RIPA lysis buffer, and quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). After separation by SDS-PAGE, protein samples were electrophoretically transferred to polyvinylidene difluoride membranes. These nonfat dry milk-blocked membranes were incubated with anti-PPAR $\alpha$  (1:500; Santa Cruz Biotechnology, Dallas, TX, United States), anti-CPT1A (1:1000; Santa Cruz Biotechnology), anti-SLC27A (1:1000; Santa Cruz Biotechnology), and anti-GAPDH (1:1000; Santa Cruz Biotechnology) overnight at 4 °C, and reacted with horseradish-peroxidase-conjugated secondary antibody (1:1500; Jackson ImmunoResearch Laboratories, West Grove, PA, United States) for 1 h at room temperature. Chemiluminescent signals were visualized by ECL detection system, and scanned densitometrically by Image Lab Software 5.1 software (Bio-Rad Laboratories).

### **Phenotypic evaluation for hepatocellular steatosis**

According to the above-mentioned subgrouping, HepG2 cells in normal, steatosis, control, circRNA, circRNA+mimics, and circRNA+mimics NC groups were plated on cover glasses, and fixed in 4% paraformaldehyde. After washing in distilled water, each group was treated with 0.5% Oil Red O for 30 min, 60% isopropanol for 10–20 s, and hematoxylin counterstaining for 10–20 s so as to uncover the lipid droplets with neutral fat (triglyceride (TG)). Those HepG2 cells with cytoplasmic enrichment of positive-staining droplets were defined to be steatotic. Quantitatively, the TG level in each group was enzymatically analyzed using the TG Assay Kit (Applygen Technologies, Shanghai, China)<sup>[35]</sup>. Lysed HepG2 cells were subjected to centrifugation at 12000 rpm for 5 min, followed by coculture of supernatant and working solution for 10 min at 37 °C. OD<sub>550</sub> indicated the hepatocellular TG concentration, which was normalized against the protein content of HepG2 cells evaluated by BCA method.

### **Statistical analysis**

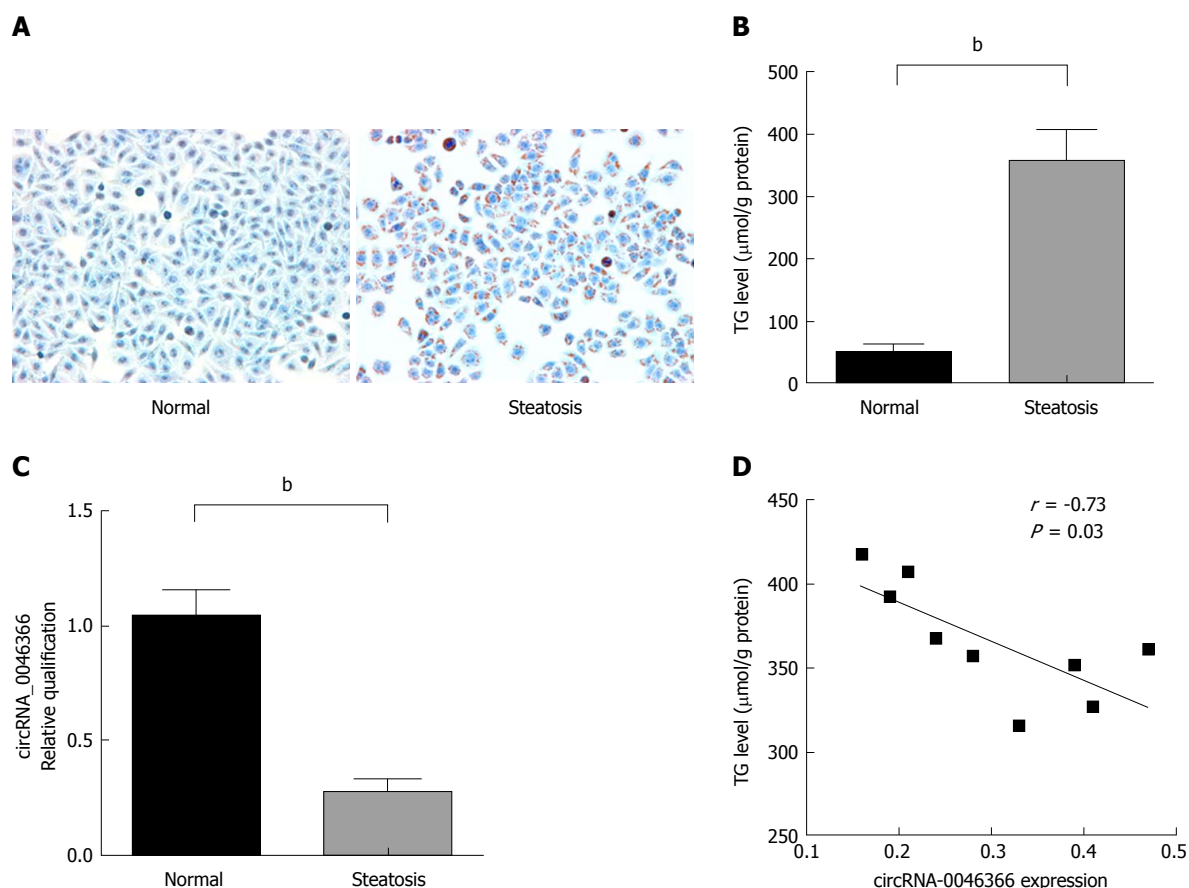
The present results are expressed as mean  $\pm$  SD. Statistical analysis was performed by Student's *t*-test or one-way analysis of variance with GraphPad Prism Software (GraphPad, La Jolla, CA, United States)<sup>[36]</sup>. Fisher's exact test was used to filter the significant pathways using R software 3.3.1 (R Development Core Team, Vienna, Austria). Differences with *P* < 0.05 (two-tailed) were considered statistically significant.

## **RESULTS**

### **circRNA\_0046366 deficiency characterized high fat-induced hepatocellular steatosis**

In contrast to the steatosis-free phenotype of the normal group, enrichment of lipid droplets dominated the steatosis group. Oil Red O staining specific to lipid droplets verified accumulation of neutral fat (TG) under FFA stimulation (Figure 1A). Consistent with these





**Figure 1** circRNA\_0046366 loss demonstrates the epigenetic characteristic of FFA-induced hepatocellular steatosis. A: Oil Red O staining identified HepG2 cells with (steatosis group) or without (normal group) FFA-induced steatosis (200  $\times$ ); B: The steatosis group exhibited significant up-regulation of TG content; C: circRNA\_0046366 quantification revealed inhibition of its expression after hepatocellular steatosis; D: circRNA\_0046366 expression was inversely correlated with hepatocellular TG level in the steatosis group. Results are expressed as mean  $\pm$  SD. <sup>b</sup> $P < 0.01$ . FFA: Free fatty acid; TG: Triglyceride.

pathological observations, an enzymatic assay showed that the TG level of the steatosis group was higher than that of the normal group (Figure 1B). Compared to the normal group, the steatosis group exhibited significant loss of circRNA\_0046366 expression (Figure 1C). FASN, which shares the same precursor mRNA (pre-mRNA) with circRNA\_0046366, showed much higher mRNA level ( $7.64 \pm 0.54$ ) in the steatosis group in comparison to that of the normal group ( $0.98 \pm 0.06$ ,  $P < 0.0001$ ). An inverse correlation between circRNA\_0046366 expression and hepatocellular TG level ( $r = -0.73$ ,  $P = 0.03$ ; Figure 1D) suggested an essential role in the occurrence of hepatocellular steatosis.

#### circRNA\_0046366 functioned as antagonist of miR-34a

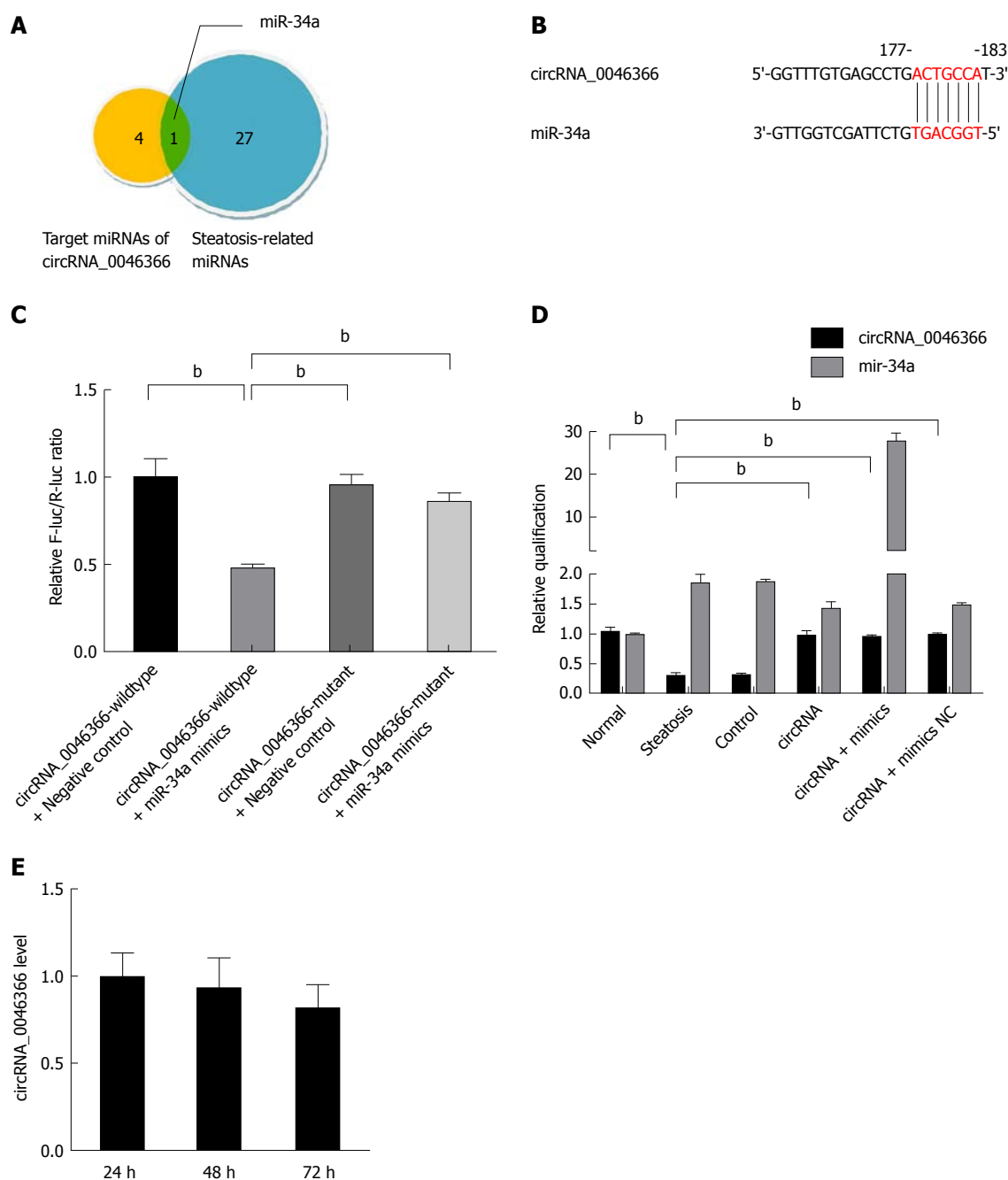
Five miRNAs (miR-34a, miR-513a-5p, miR-646, miR-892a and miR-1265) were predicted to be the targets of circRNA\_0046366, using the algorithms of base complementation between MRE of circRNA and seed sequence of miRNA (Figure 2A). The intersection of circRNA\_0046366-targeted miRNAs and hepatic-steatosis-related miRNAs recognized those that mediated the regulatory effect of circRNA\_0046366 on hepatic steatogenesis. miR-34a was the only target

of circRNA\_0046366 and had a critical role in hepatocellular lipid dysmetabolism (Figure 2A).

Dual-luciferase reporter assay was performed to verify the MRE-based circRNA-miRNA interaction (Figure 2B). After cotransfection of pMIR-REPORT vector containing wild-type circRNA\_0046366 and miR-34a mimics, the activity ratio of firefly/Renilla luciferases was down-regulated in the pMIR-REPORT-circRNA\_0046366-wildtype-treated group (Figure 2C). On the contrary, there was no reduction in relative luciferase activity in the pMIR-REPORT-circRNA\_0046366-mutant-treated group with the MRE-lacking vector (Figure 2C). This suggests that circRNA\_0046366 is an antagonist of miR-34a via targeted, complementary binding.

#### circRNA\_0046366 up-regulation rescued PPAR $\alpha$ expression via miR-34a inactivation

HepG2 cells were exposed to transfection of circRNA\_0046366-carrying expression vector, which led to normalization of circRNA level, on the condition of FFA culture (Figure 2D). miR-34a was inactivated by circRNA\_0046366-dependent antagonism. Because of the approximately stable level of intracellular circRNA\_0046366 (Figure 2E), pharmacological effect



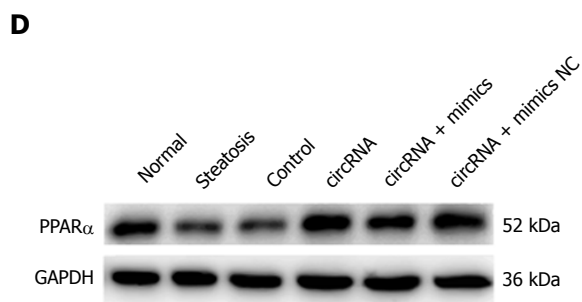
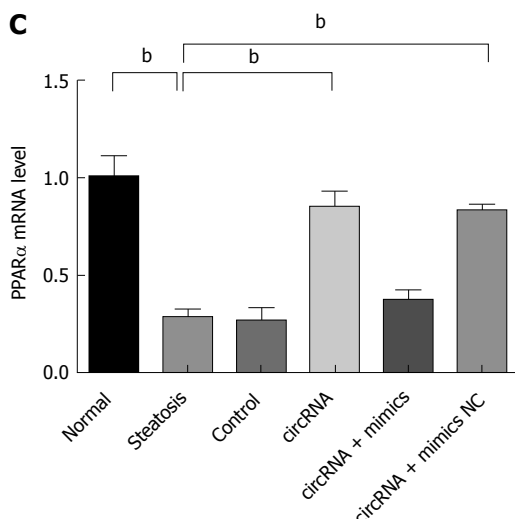
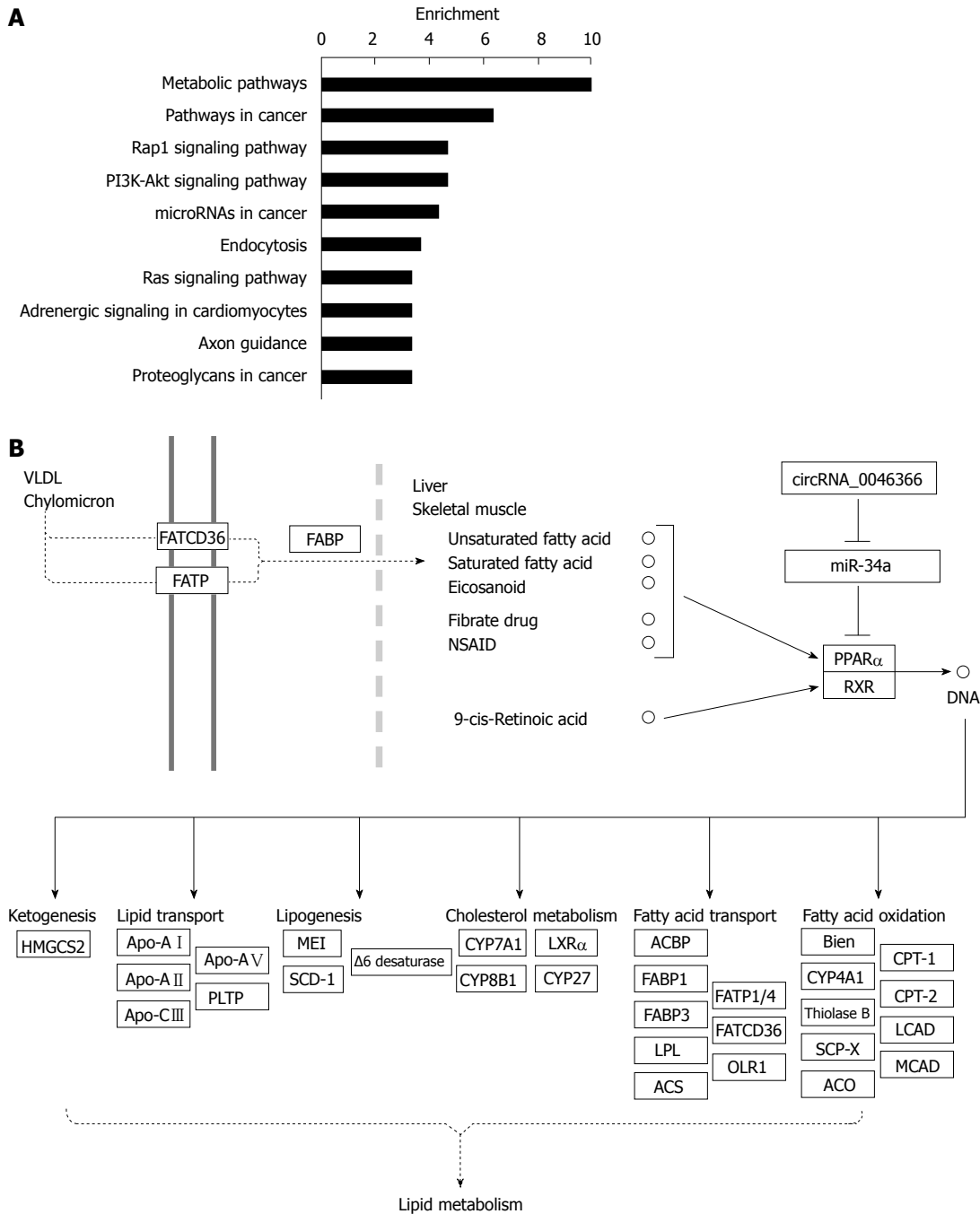
**Figure 2** circRNA\_0046366 functions as the antagonist of miR-34a. A: Set intersection filters circRNA\_0046366-targeting miRNAs associated with hepatocellular steatosis; B: circRNA-miRNA interaction recognized by the base complementation between MRE of circRNA\_0046366 and seed sequence of miR-34a; C: Dual-luciferase reporter assay verified the antagonistic effect of circRNA\_0046366 on miR-34a; D: Treatment of circRNA-carrying vector normalized expression level of circRNA\_0046366 with counteracting impact on miR-34a; E: Results are expressed as mean  $\pm$  SD. <sup>b</sup> $P < 0.01$ .

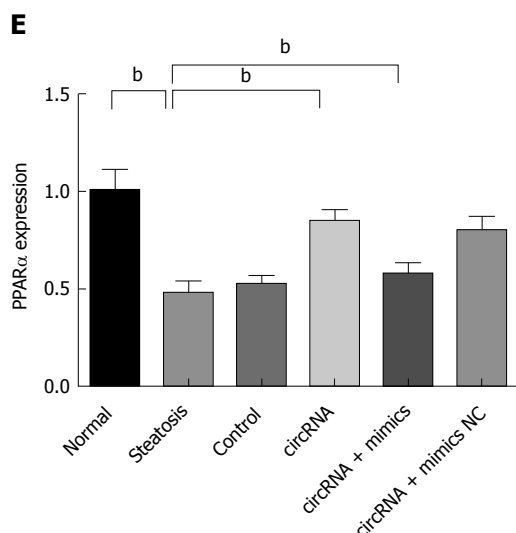
could be yielded from the miR-34a inactivation.

Bioinformatic analysis showed that miR-34a had a major impact on metabolic signaling pathways (hsa01100), pathways in cancer (hsa05200), Rap1 signaling pathway (hsa04015), PI3K/Akt signaling pathway (hsa04151), and miRNAs in cancer (hsa05206) (Figure 3A). Some of these metabolic pathways were shown to mediate the steatosis-inducing action of miR-34a. Gene interaction analysis subsequently revealed that PPAR $\alpha$ , the high-affinity target of miR-34a, controlled multiple lipometabolic genes by transcriptional induction (Figure

3B). Thus, circRNA\_0046366 is proposed to modulate hepatocellular steatosis through the miR-34a/PPAR $\alpha$  regulatory system.

miR-34a-induced PPAR $\alpha$  inhibition was one of the critical manifestations of hepatocellular steatosis (Figure 3C and D). A significant increase in PPAR $\alpha$ , at both transcriptional and translational levels, characterized the circRNA and circRNA+mimics NC groups with circRNA\_0046366 normalization (Figure 3C and D). However, saturated binding of circRNA\_0046366 and miR-34a prevented PPAR $\alpha$  restoration in the





**Figure 3** circRNA\_0046366 restoration rescued PPAR $\alpha$  expression by miR-34a inactivation. A: Metabolic pathway (hsa01100) was the top-enriched pathway that was affected by circRNA\_0046366-induced miR-34a antagonism; B: PPAR $\alpha$  mediated the regulatory role of miR-34a in lipid metabolism signaling by controlling expression of multiple lipometabolic genes (adapted from the KEGG database). miR-34a inactivation leads to the up-regulated expression of PPAR $\alpha$  at transcriptional (C) and translational (D) levels. Results are expressed as mean  $\pm$  SD. <sup>b</sup> $P < 0.01$ . KEGG: Kyoto Encyclopedia of Genes and Genomes; PPAR: Peroxisome proliferator-activated receptor.

circRNA+mimics group (Figure 3C and D). These findings showed that circRNA\_0046366 rescued PPAR $\alpha$  expression, mainly by abolishing the inhibitory effect of miR-34a.

#### PPAR $\alpha$ restoration promoted transcriptional activation of lipometabolic genes

Resulting from the circRNA\_0046366 loss, PPAR $\alpha$  repression took place in steatotic cells upon miR-34a activation. The PPAR $\alpha$  deficiency abrogated its transcriptional regulation of multiple genes associated with lipid metabolism, especially CPT1A and SLC27A (Figure 4A-I). Their abnormal expression contributed to the lipometabolic imbalance, which was largely responsible for the occurrence of hepatocellular steatosis.

In contrast to the abnormal transcription of downstream genes after FFA exposure (steatosis, control, and circRNA+mimics groups), circRNA\_0046366-based PPAR $\alpha$  restoration brought about improvement in their expression in both circRNA and circRNA+mimics NC groups (Figure 4B-H). TG metabolic genes (CPT1A and SLC27A) among these exhibited significant up-regulation of mRNA levels (Figure 4G and H). Consequently, the transcription-promoting effect of PPAR $\alpha$  gave rise to obvious elevation of hepatocellular CPT1A and SLC27A, with similar levels to those in the normal group (Figure 4I). Thereby, a resolution of the steatosis-related lipid dysmetabolism could be achieved.

#### Improvement of lipid metabolism ameliorated hepatocellular steatosis by TG reduction

Biochemical analysis highlighted an overview of the lipometabolic improvements that were in agreement

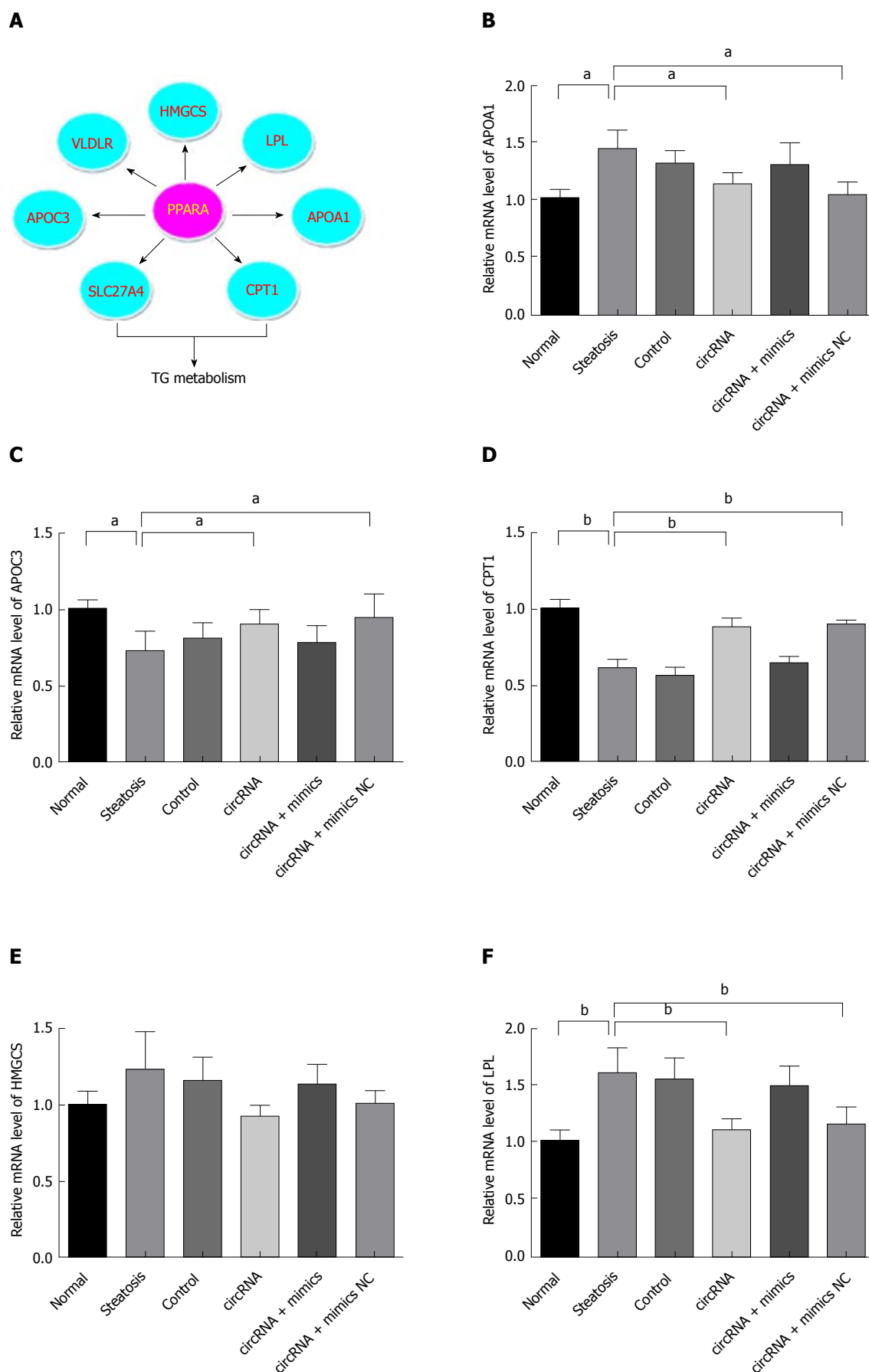
with the gene expression. Compared to the normal group, reduction of hepatocellular TG level dominated the groups with regaining of lipid homeostasis (steatosis group vs circRNA group:  $368.73 \pm 46.16 \mu\text{mol/g}$  vs  $215.13 \pm 26.42 \mu\text{mol/g}$  protein,  $P < 0.01$ ; steatosis group vs circRNA+mimics NC group:  $368.73 \pm 46.16 \mu\text{mol/g}$  vs  $227.40 \pm 54.06 \mu\text{mol/g}$  protein,  $P < 0.05$ ) (Figure 4J).

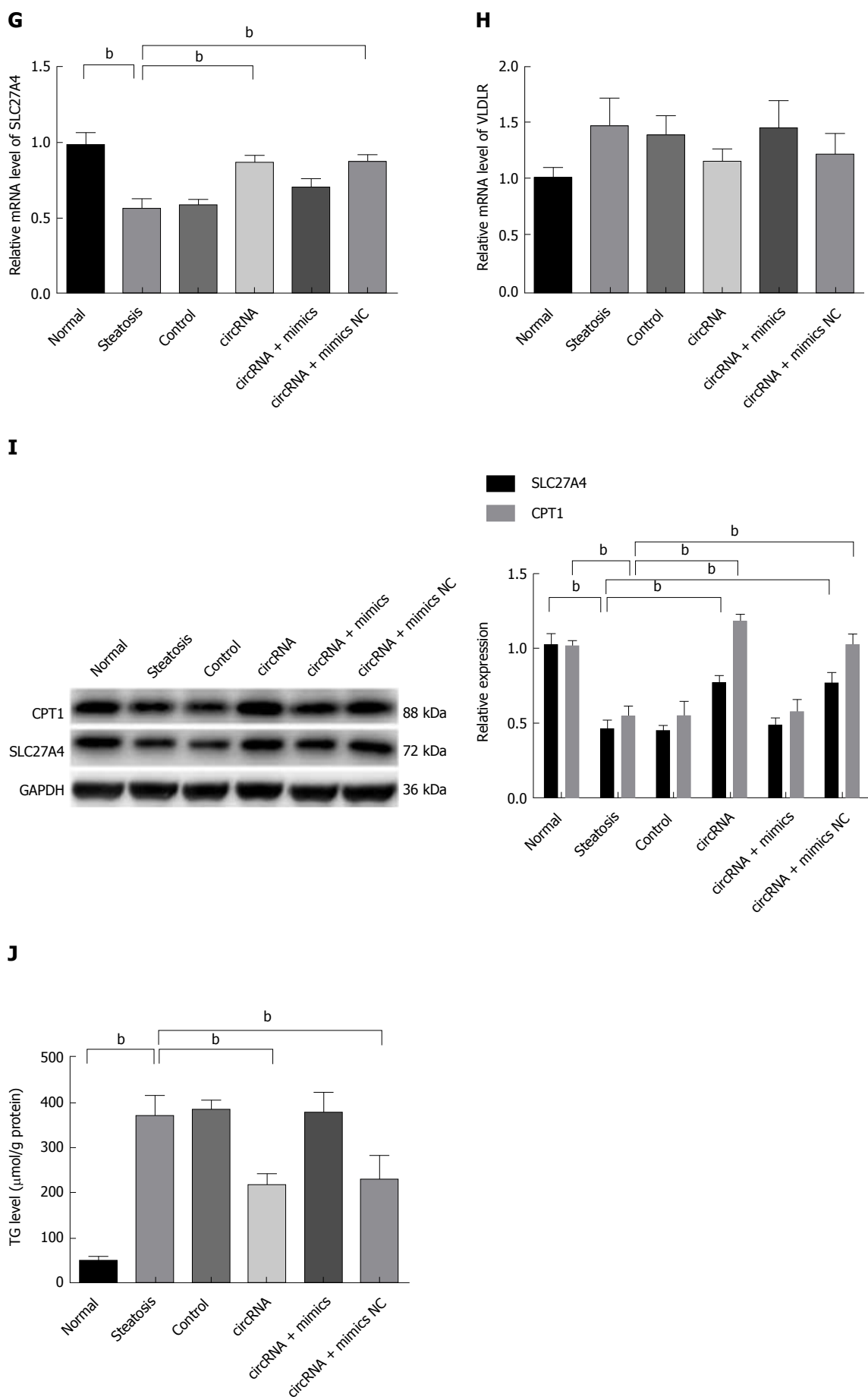
Pathophysiological alleviation was achieved from disruption of TG deposition. The groups with diminished TG content (circRNA and circRNA+mimics NC), instead of those without alteration (steatosis, control, and circRNA+mimics), lacked cytoplasmic lipid droplets under treatment with oleate and palmitate (Figure 4K). Therefore, attenuation of hepatocellular steatosis was verified after circRNA\_0046366-based miR-34a antagonism and restoration of lipid metabolism *via* the miR-34a-related PPAR signaling.

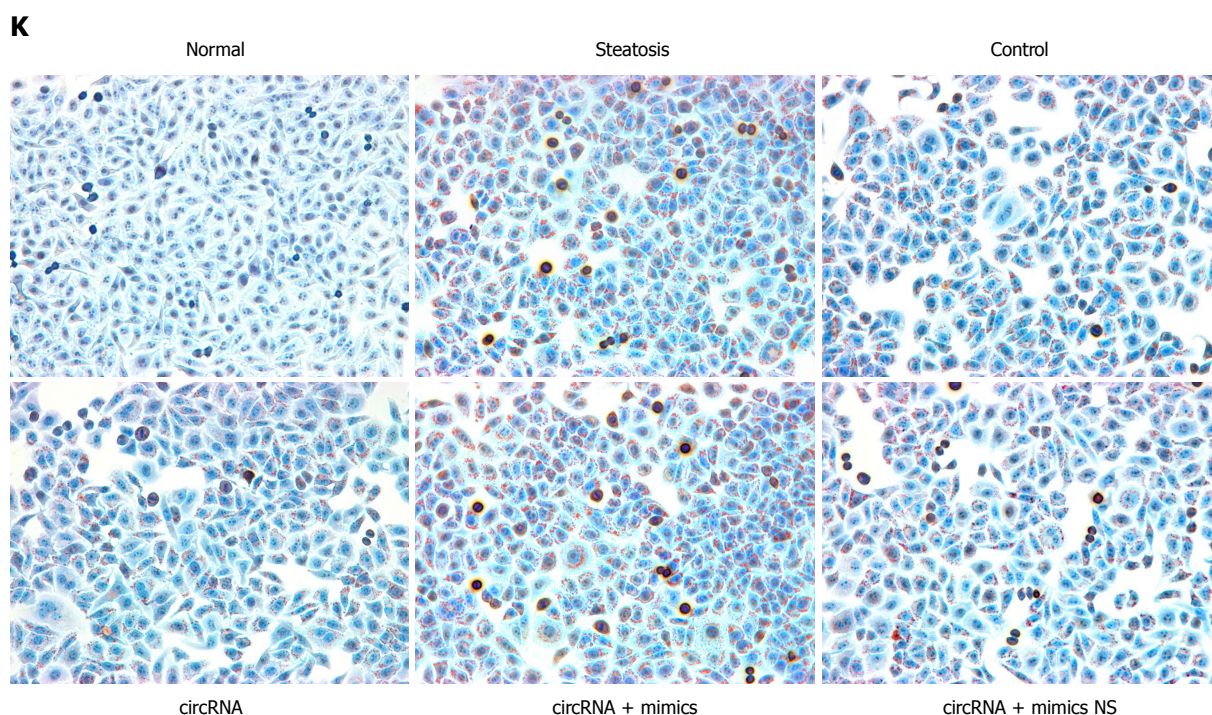
## DISCUSSION

circRNA has recently been discovered to serve as a novel, yet crucial, component of epigenetic regulation of miRNA-mRNA interaction<sup>[14]</sup>. Its antagonistic effect against miRNA is one of its most important mechanisms related to physiological functions (e.g., insulin production and cartilage degradation)<sup>[37,38]</sup> and various diseases (e.g., Alzheimer's disease, colorectal cancer, ischemia-reperfusion injury, and hypertrophic heart failure)<sup>[24,34,39-41]</sup>. In the present study, prominent loss of circRNA\_0046366 expression characterized the steatotic HepG2 cells under stimulation with saturated (palmitate) and polyunsaturated (oleate) fatty acids. In contrast, the mRNA level of FASN, one of the major determinants in *de novo* lipogenesis<sup>[42]</sup>, experienced obvious up-









**Figure 4** PPAR $\alpha$  normalization improved hepatocellular steatosis by transcriptional promotion of lipometabolic genes. A: PPAR $\alpha$  restoration by circRNA-0046366 promoted expression of CPT1A and SLC27A at the transcriptional level; B: Transcriptional activation of CPT1A and SLC27A resulted in their significant up-regulation. Improved expression of lipometabolic genes reduced the TG level (C) and attenuated hepatocellular steatosis (D) (200  $\times$ ). Results are expressed as mean  $\pm$  SD. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ . PPAR: Peroxisome proliferator-activated receptor; TG: Triglyceride.

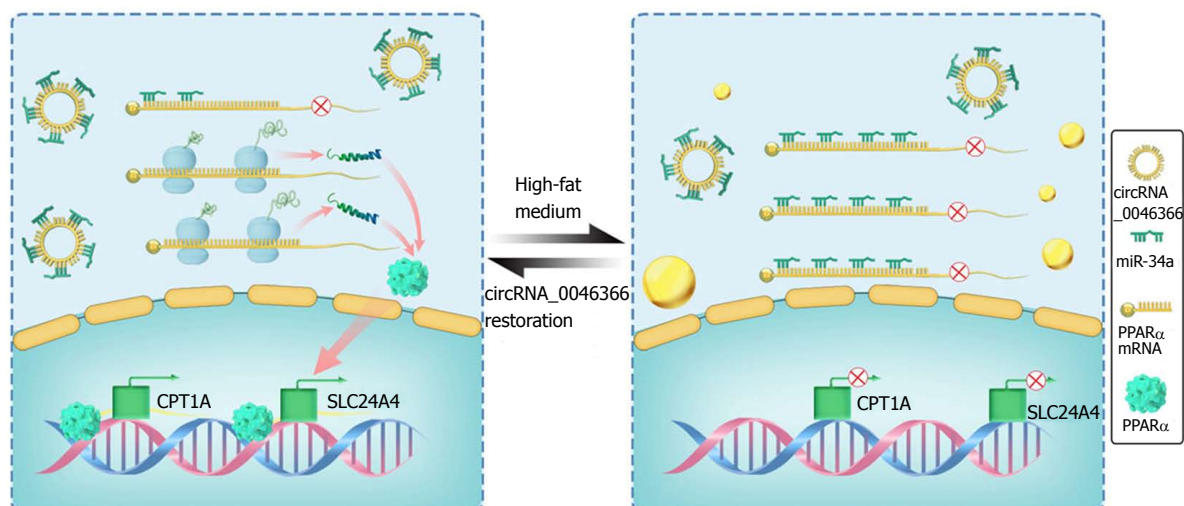
regulation on condition of fatty acids exposure. Being the products of alternative splicing, circRNA\_0046366 and FASN share a common pre-mRNA. Thus, increase in FASN transcription hampers the circRNA\_0046366 production by the competitive effect in alternative splicing. Some surprising results even confirmed that circRNA\_0046366 level significantly correlated with total TG content in an inverse manner. circRNA\_0046366 dysregulation is, therefore, suggested to play an essential, perhaps miRNA-antagonizing, role in FFA-induced hepatocellular steatosis.

To investigate the actions of circRNA\_0046366 underlying hepatocellular steatosis, target prediction and key-miRNA mining were performed on the basis of base-complementation algorithms and miRNA-set intersection, respectively<sup>[28,43]</sup>. miR-34a is a well-established miRNA with lipogenic properties<sup>[20]</sup>, and it was the only candidate target of circRNA\_0046366 that was associated with steatosis-related lipid dysmetabolism. Dual-luciferase reporter assay provided further evidence for the circRNA\_0046366/miR-34a interaction. In contrast to cotransfection of miR-34a with reporter vector containing mutant circRNA\_0046366, miR-34a demonstrated a complementary effect on wild-type circRNA\_0046366 by a decrease in firefly/Renilla luciferase activity. The results of bioinformatic and functional analysis highlight that circRNA\_0046366 is a miR-34a-specific antagonist. Because of its multiple-targeted characteristics, miR-34 facilitates a subtle antagonism of steatosis-related miRNA/mRNA interaction by relative limited circRNA\_0046366 on a

basis of competitive binding<sup>[44-46]</sup>.

Targetome and targetome-based pathway analyses of miR-34a were integrated to uncover the downstream signaling and related functions of circRNA\_0046366 in a miRNA-dependent way<sup>[47,48]</sup>. As a result of the top-ranking enrichment, metabolic pathway hsa01100 was identified as a key signaling pathway that was influenced by circRNA\_0046366. circRNA\_0046366 focuses its impact on metabolic process, especially lipid metabolism. PPAR $\alpha$ , a transcription factor of the NR1C nuclear receptor subfamily, has recently been identified as a direct target of miR-34a<sup>[20]</sup>. This kind of liver-specific, ligand-activated isoform of the PPAR family induces the expression of multiple genes with lipometabolic characteristics<sup>[29]</sup>. Lack of PPAR $\alpha$  in obese patients confers a high risk of insulin resistance, n-3 long-chain polyunsaturated fatty acid depletion, and liver steatogenesis<sup>[49]</sup>. Increasing evidence suggests a miR-34a/PPAR $\alpha$  regulatory system, which acts as the crucial mediator of the actions of circRNA\_0046366 in hepatocellular lipid metabolism (Figure 5).

With the relative stability in expressive dynamics, normalized circRNA\_0046366 brought about antagonistic inactivation of miR-34a in the circRNA and circRNA+mimics NC groups in our study. PPAR $\alpha$  up-regulation within these groups occurred as a result of diminished miR-34a-induced gene inhibition. This counteracting role of circRNA\_0046366 against the miR-34a/PPAR $\alpha$  regulatory system is supposed to expressively activate the lipometabolic genes and, in turn, rescue hepatic lipid homeostasis. Indeed,



**Figure 5** Schematic representation exhibits the circRNA\_0046366/miR-34a/PPAR $\alpha$  axis underlying occurrence and resolution of hepatocellular steatosis. In contrast to the hepatic steatogenesis that occurs upon its deficiency, circRNA\_0046366 restoration abrogates the inhibitory effect of miR-34a on PPAR $\alpha$  by antagonizing the miRNA-mRNA interaction. PPAR $\alpha$  normalization transcriptionally activates the lipometabolic genes, which further ameliorates hepatic steatosis by regaining lipid homeostasis. mi: Micro; PPAR: Peroxisome proliferator-activated receptor.

circRNA\_0046366 treatment dramatically increased both CPT1A and SLC27A, the key genes downstream to PPAR $\alpha$ <sup>[50]</sup>, at transcriptional and translational levels.

CPT1a is located on the liver mitochondrial outer membrane and forms a hexamer fatty acid transfer complex<sup>[51]</sup>, and functions as the rate-limiting enzyme of fatty acid  $\beta$ -oxidation<sup>[52]</sup>. CPT1A deficiency gives rise to impairment of long-chain fatty acid oxidation and ketogenesis, which are involved in the occurrence of hepatic steatosis<sup>[53,54]</sup>. In contrast to the insufficient expression of CPT1A during steatogenesis, PPAR $\alpha$  agonists reduce lipid accumulation in close association with up-regulated CPT1A concentration<sup>[55]</sup>. SLC27A, also known as fatty acid transport protein (FATP)4, reflects another liver-dominant, evolutionarily conserved PPAR $\alpha$  target that takes central place in the cellular uptake and metabolism of long-chain and very long-chain fatty acids<sup>[56,57]</sup>. Lowered SLC27A level facilitates fat deposition *via* the reduction of CPT1A-mediated fatty acid oxidation<sup>[58]</sup>. A similar phenomenon has been verified in FATP4-deficient animals with aggravated liver fatty degeneration under high-fat diet<sup>[59]</sup>. Therefore, restoration of CPT1A and SLC27A seen in the present study could counterbalance lipid metabolism by increasing activity of lipolytic catalyzation.

As defined by the colorimetric detection assay, regaining of lipid homeostasis with reduced intracellular TG content was validated in groups with expression-rescued lipolytic enzymes (circRNA and circRNA+mimics NC) compared with those without enough catalytic lipid oxidation (steatosis, control and circRNA+mimics). Disruption of glycerol-esterified neutral fat accumulation improved the pathophysiological outcome in conditions of high-fat culture. In contrast to those in the TG-enriched groups, HepG2 cells in the circRNA and circRNA+mimics NC groups showed a dramatic decrease in cytoplasmic

lipid droplets in parallel with TG down-regulation. Finally, they were protected from lipotoxicity-stimulated phenotypic transition toward hepatocellular steatosis.

## ARTICLE HIGHLIGHTS

### Research background

Hepatic steatosis reflects one of the most common chronic liver diseases with hepatocyte-specific lipid dysmetabolism and triglyceride (TG) accumulation. Its close association to steatohepatitis, metabolic syndrome, and extrahepatic diseases (*i.e.*, cardiovascular events, cerebrovascular diseases, cancers) indicates an importance for clinical interference. Micro (mi)R-34a is now confirmed to underlie the hepatic steatosis. However, the ambiguity in miR-34a-specific antagonist keeps hepatic steatosis from effective therapy. Circular (circ)RNA has recently been determined to interact with miRNA, mainly on the basis of complementation between miRNA response element (MRE) of circRNA and 'seed sequence' of miRNA. This circRNA/miRNA interaction abolishes the inhibitory effect of miRNA on its targets. circRNA, therefore, is highlighted to function in a miRNA-antagonizing manner.

### Research motivation

Because of its importance in hepatic steatosis, miR-34a represents a critical target of clinical intervention. miR-34a-targeting antagonist, therefore, is assessed in our experiments so as to cure hepatosteatotic degeneration on the basis of miR-34a inactivation.

### Research objectives

Serving as the selective sponge of miRNA, circRNA is exposed to bioinformatical and functional analysis for a purpose of uncovering the antagonist specific to miR-34a.

### Research methods

To shed light on the antagonistic effect of circRNA against miR-34a, investigation of miR-34a-targeting circRNA was carried out by MRE recognition and dual-luciferase reporter assay. The filtered circRNA was then subjected to functional study in HepG2-based experimental steatosis induced high-fat stimulation. In detail, rescue experiment, real-time quantitative PCR, and western blot demonstrated the impact of circRNA on miR-34a, peroxisome proliferator-activated receptor (PPAR) $\alpha$ , and transcriptional downstream genes. Both triglyceride (TG) quantification and cytopathologic assessment revealed



the steatosis-related outcome of circRNA administration.

## Research results

circRNA\_0046366 loss reflects the epigenetic characteristics of high fat-induced hepatocellular steatosis. Both bioinformatical and functional proofs indicate a circRNA\_0046366-dependent miR-34a inactivation by the complementary antagonism. Dramatically, circRNA\_0046366 up-regulation abolishes the inhibitory effect of miR-34a on PPAR $\alpha$ . PPAR $\alpha$  restoration further promotes the transcriptional activity of downstream genes, which improves the steatosis-related TG metabolism. In conclusion, the circRNA\_0046366 administration leads to a significant attenuation of TG accumulation, and finally alleviates the hepatosteatotic phenotype with decreased cytoplasmic lipid droplets.

## Research conclusions

The present study identifies great importance of circRNA\_0046366/miR-34a/PPAR $\alpha$  signaling in hepatocellular steatosis. circRNA\_0046366 may act as a potential agent in the clinical interference of hepatic steatosis.

## Research perspectives

The role of circRNA\_0046366 in hepatocellular steatosis qualifies it for further evaluation in experimental hepatic steatosis with different etiologies (*i.e.*, high-fat high-cholesterol diet, high-fat high-fructose diet, methionine and choline-deficient diet). These results could provide substantial evidence for circRNA\_0046366-related prevention and therapy of hepatic steatosis.

## REFERENCES

- Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231 [PMID: 11961152 DOI: 10.1056/NEJMra011775]
- Wang FS, Fan JG, Zhang Z, Gao B, Wang HY. The global burden of liver disease: the major impact of China. *Hepatology* 2014; **60**: 2099-2108 [PMID: 25164003 DOI: 10.1002/hep.27406]
- Williams CD, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, Landt CL, Harrison SA. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology* 2011; **140**: 124-131 [PMID: 20858492 DOI: 10.1053/j.gastro.2010.09.038]
- Caballería L, Pera G, Auladell MA, Torán P, Muñoz L, Miranda D, Alumà A, Casas JD, Sánchez C, Gil D, Aubà J, Tibau A, Canut S, Bernad J, Aizpurua MM. Prevalence and factors associated with the presence of nonalcoholic fatty liver disease in an adult population in Spain. *Eur J Gastroenterol Hepatol* 2010; **22**: 24-32 [PMID: 19730384 DOI: 10.1097/MEG.0b013e32832fcd0f]
- Fung J, Lee CK, Chan M, Seto WK, Lai CL, Yuen MF; Hong Kong Liver Health Census Study Group. High prevalence of non-alcoholic fatty liver disease in the Chinese - results from the Hong Kong liver health census. *Liver Int* 2015; **35**: 542-549 [PMID: 24923704 DOI: 10.1111/liv.12619]
- Goh GB, Kwan C, Lim SY, Venkatanarasimha NK, Abu-Bakar R, Krishnamoorthy TL, Shim HH, Tay KH, Chow WC. Perceptions of non-alcoholic fatty liver disease - an Asian community-based study. *Gastroenterol Rep (Oxf)* 2016; **4**: 131-135 [PMID: 26463276 DOI: 10.1093/gastro/gov047]
- Calzadilla Bertot L, Adams LA. The Natural Course of Non-Alcoholic Fatty Liver Disease. *Int J Mol Sci* 2016; **17**: pii: E774 [PMID: 27213358 DOI: 10.3390/ijms17050774]
- Ascha MS, Hanounah IA, Lopez R, Tamimi TA, Feldstein AF, Zein NN. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *Hepatology* 2010; **51**: 1972-1978 [PMID: 20209604 DOI: 10.1002/hep.23527]
- Marchesini G, Marzocchi R. Metabolic syndrome and NASH. *Clin Liver Dis* 2007; **11**: 105-117, ix [PMID: 17544974 DOI: 10.1016/j.cld.2007.02.013]
- Richard J, Lingvay I. Hepatic steatosis and Type 2 diabetes: current and future treatment considerations. *Expert Rev Cardiovasc Ther* 2011; **9**: 321-328 [PMID: 21438811 DOI: 10.1586/erc.11.15]
- Gaggini M, Morelli M, Buzzigoli E, DeFronzo RA, Bugianesi E, Gastaldelli A. Non-alcoholic fatty liver disease (NAFLD) and its connection with insulin resistance, dyslipidemia, atherosclerosis and coronary heart disease. *Nutrients* 2013; **5**: 1544-1560 [PMID: 23666091 DOI: 10.3390/nu5051544]
- Mikolasevic I, Orlic L, Stimac D, Hrstic I, Jakopcic I, Milic S. Non-alcoholic fatty liver disease and colorectal cancer. *Postgrad Med J* 2017; **93**: 153-158 [PMID: 27852946 DOI: 10.1136/postgradmedj-2016-134383]
- Tutar Y. miRNA and cancer; computational and experimental approaches. *Curr Pharm Biotechnol* 2014; **15**: 429 [PMID: 25189575]
- Mitra CK, Korla K. Functional, structural, and sequence studies of microRNA. *Methods Mol Biol* 2014; **1107**: 189-206 [PMID: 24272438 DOI: 10.1007/978-1-62703-748-8\_11]
- Feng YY, Xu XQ, Ji CB, Shi CM, Guo XR, Fu JF. Aberrant hepatic microRNA expression in nonalcoholic fatty liver disease. *Cell Physiol Biochem* 2014; **34**: 1983-1997 [PMID: 25562147 DOI: 10.1159/000366394]
- Pogribny IP, Starlard-Davenport A, Tryndyak VP, Han T, Ross SA, Rusyn I, Beland FA. Difference in expression of hepatic microRNAs miR-29c, miR-34a, miR-155, and miR-200b is associated with strain-specific susceptibility to dietary nonalcoholic steatohepatitis in mice. *Lab Invest* 2010; **90**: 1437-1446 [PMID: 20548288 DOI: 10.1038/labinvest.2010.113]
- Liu XL, Pan Q, Zhang RN, Shen F, Yan SY, Sun C, Xu ZJ, Chen YW, Fan JG. Disease-specific miR-34a as diagnostic marker of non-alcoholic steatohepatitis in a Chinese population. *World J Gastroenterol* 2016; **22**: 9844-9852 [PMID: 27956809 DOI: 10.3748/wjg.v22.i44.9844]
- Yamada H, Suzuki K, Ichino N, Ando Y, Sawada A, Osakabe K, Sugimoto K, Ohashi K, Teradaira R, Inoue T, Hamajima N, Hashimoto S. Associations between circulating microRNAs (miR-21, miR-34a, miR-122 and miR-451) and non-alcoholic fatty liver. *Clin Chim Acta* 2013; **424**: 99-103 [PMID: 23727030 DOI: 10.1016/j.cca.2013.05.021]
- Salvoza NC, Klinzing DC, Gopez-Cervantes J, Baclig MO. Association of Circulating Serum miR-34a and miR-122 with Dyslipidemia among Patients with Non-Alcoholic Fatty Liver Disease. *PLoS One* 2016; **11**: e0153497 [PMID: 27077736 DOI: 10.1371/journal.pone.0153497]
- Ding J, Li M, Wan X, Jin X, Chen S, Yu C, Li Y. Effect of miR-34a in regulating steatosis by targeting PPAR $\alpha$  expression in nonalcoholic fatty liver disease. *Sci Rep* 2015; **5**: 13729 [PMID: 26330104 DOI: 10.1038/srep13729]
- Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. *Nature* 2013; **495**: 384-388 [PMID: 23446346 DOI: 10.1038/nature11993]
- Tang CM, Zhang M, Huang L, Hu ZQ, Zhu JN, Xiao Z, Zhang Z, Lin QX, Zheng XL, -Yang M, Wu SL, Cheng JD, Shan ZX. CircRNA\_000203 enhances the expression of fibrosis-associated genes by derepressing targets of miR-26b-5p, Col1a2 and CTGF, in cardiac fibroblasts. *Sci Rep* 2017; **7**: 40342 [PMID: 28079129 DOI: 10.1038/srep40342]
- Zhou B, Yu JW. A novel identified circular RNA, circRNA\_010567, promotes myocardial fibrosis via suppressing miR-141 by targeting TGF- $\beta$ 1. *Biochem Biophys Res Commun* 2017; **487**: 769-775 [PMID: 28412345 DOI: 10.1016/j.bbrc.2017.04.044]
- Wang K, Long B, Liu F, Wang JX, Liu CY, Zhao B, Zhou LY, Sun T, Wang M, Yu T, Gong Y, Liu J, Dong YH, Li N, Li PF. A circular RNA protects the heart from pathological hypertrophy and heart failure by targeting miR-223. *Eur Heart J* 2016; **37**: 2602-2611 [PMID: 26802132 DOI: 10.1093/eurheartj/ehv713]
- Gómez-Lechón MJ, Donato MT, Martínez-Romero A, Jiménez N, Castell JV, O'Connor JE. A human hepatocellular in vitro model to investigate steatosis. *Chem Biol Interact* 2007; **165**: 106-116 [PMID: 17188672 DOI: 10.1016/j.cbi.2006.11.004]
- Dudekula DB, Panda AC, Grammatikakis I, De S, Abdelmohsen K, Gorospe M. CircInteractome: A web tool for exploring circular

- 26 RNAs and their interacting proteins and microRNAs. *RNA Biol* 2016; **13**: 34-42 [PMID: 26669964 DOI: 10.1080/15476286.2015.1128065]
- 27 Sobolewski C, Calo N, Portius D, Foti M. MicroRNAs in fatty liver disease. *Semin Liver Dis* 2015; **35**: 12-25 [PMID: 25632931 DOI: 10.1055/s-0034-1397345]
- 28 Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* 2006; **34**: D140-D144 [PMID: 16381832 DOI: 10.1093/nar/gkj112]
- 29 Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M. KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res* 2012; **40**: D109-D114 [PMID: 22080510 DOI: 10.1093/nar/gkr988]
- 30 Yi M, Horton JD, Cohen JC, Hobbs HH, Stephens RM. WholePathwayScope: a comprehensive pathway-based analysis tool for high-throughput data. *BMC Bioinformatics* 2006; **7**: 30 [PMID: 16423281 DOI: 10.1186/1471-2105-7-30]
- 31 Marriotti AS, Vasieva O, Fang Y, Copeland NA, McLennan AG, Jones NJ. NUDT2 Disruption Elevates Diadenosine Tetraphosphate (Ap4A) and Down-Regulates Immune Response and Cancer Promotion Genes. *PLoS One* 2016; **11**: e0154674 [PMID: 27144453 DOI: 10.1371/journal.pone.0154674]
- 32 Yang HM, Do HJ, Kim DK, Park JK, Chang WK, Chung HM, Choi SY, Kim JH. Transcriptional regulation of human Oct4 by steroidogenic factor-1. *J Cell Biochem* 2007; **101**: 1198-1209 [PMID: 17226773 DOI: 10.1002/jcb.21244]
- 33 Li Z, Huang C, Bao C, Chen L, Lin M, Wang X, Zhong G, Yu B, Hu W, Dai L, Zhu P, Chang Z, Wu Q, Zhao Y, Jia Y, Xu P, Liu H, Shan G. Exon-intron circular RNAs regulate transcription in the nucleus. *Nat Struct Mol Biol* 2015; **22**: 256-264 [PMID: 25664725 DOI: 10.1038/nsmb.2959]
- 34 Lin SP, Ye S, Long Y, Fan Y, Mao HF, Chen MT, Ma QJ. Circular RNA expression alterations are involved in OGD/R-induced neuron injury. *Biochem Biophys Res Commun* 2016; **471**: 52-56 [PMID: 26845359 DOI: 10.1016/j.bbrc.2016.01.183]
- 35 Zhang XQ, Pan Y, Yu CH, Xu CF, Xu L, Li YM, Chen WX. PDIA3 Knockdown Exacerbates Free Fatty Acid-Induced Hepatocyte Steatosis and Apoptosis. *PLoS One* 2015; **10**: e0133882 [PMID: 26214517 DOI: 10.1371/journal.pone.0133882]
- 36 Upreti D, Pathak A, Kung SK. Development of a standardized flow cytometric method to conduct longitudinal analyses of intracellular CD3 $\zeta$  expression in patients with head and neck cancer. *Oncol Lett* 2016; **11**: 2199-2206 [PMID: 26998149 DOI: 10.3892/ol.2016.4209]
- 37 Liu Q, Zhang X, Hu X, Dai L, Fu X, Zhang J, Ao Y. Circular RNA Related to the Chondrocyte ECM Regulates MMP13 Expression by Functioning as a MiR-136 'Sponge' in Human Cartilage Degradation. *Sci Rep* 2016; **6**: 22572 [PMID: 26931159 DOI: 10.1038/srep22572]
- 38 Xu H, Guo S, Li W, Yu P. The circular RNA Cdr1as, via miR-7 and its targets, regulates insulin transcription and secretion in islet cells. *Sci Rep* 2015; **5**: 12453 [PMID: 26211738 DOI: 10.1038/srep12453]
- 39 Zhao Y, Alexandrov PN, Jaber V, Lukiw WJ. Deficiency in the Ubiquitin Conjugating Enzyme UBE2A in Alzheimer's Disease (AD) is Linked to Deficits in a Natural Circular miRNA-7 Sponge (circRNA; ciRS-7). *Genes (Basel)* 2016; **7**: pii: E116 [PMID: 27929395 DOI: 10.3390/genes7120116]
- 40 Xie H, Ren X, Xin S, Lan X, Lu G, Lin Y, Yang S, Zeng Z, Liao W, Ding YQ, Liang L. Emerging roles of circRNA\_001569 targeting miR-145 in the proliferation and invasion of colorectal cancer. *Oncotarget* 2016; **7**: 26680-26691 [PMID: 27058418 DOI: 10.18632/oncotarget.8589]
- 41 Zheng Q, Bao C, Guo W, Li S, Chen J, Chen B, Luo Y, Lyu D, Li Y, Shi G, Liang L, Gu J, He X, Huang S. Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs. *Nat Commun* 2016; **7**: 11215 [PMID: 27050392 DOI: 10.1038/ncomms11215]
- 42 Dorn C, Riener MO, Kirovski G, Saugspier M, Steib K, Weiss TS, Gäbele E, Kristiansen G, Hartmann A, Hellerbrand C. Expression of fatty acid synthase in nonalcoholic fatty liver disease. *Int J Clin Exp Pathol* 2010; **3**: 505-514 [PMID: 20606731]
- 43 Glažar P, Papavasileiou P, Rajewsky N. circBase: a database for circular RNAs. *RNA* 2014; **20**: 1666-1670 [PMID: 25234927 DOI: 10.1261/rna.043687.113]
- 44 Li WQ, Chen C, Xu MD, Guo J, Li YM, Xia QM, Liu HM, He J, Yu HY, Zhu L. The rno-miR-34 family is upregulated and targets ACSL1 in dimethylnitrosamine-induced hepatic fibrosis in rats. *FEBS J* 2011; **278**: 1522-1532 [PMID: 21366874 DOI: 10.1111/j.1742-4658.2011.08075.x]
- 45 Koufaris C, Wright J, Currie RA, Gooderham NJ. Hepatic microRNA profiles offer predictive and mechanistic insights after exposure to genotoxic and epigenetic hepatocarcinogens. *Toxicol Sci* 2012; **128**: 532-543 [PMID: 22584684 DOI: 10.1093/toxsci/kfs170]
- 46 Pok S, Wen V, Shackel N, Alsop A, Pyakurel P, Fahrner A, Farrell GC, Teoh NC. Cyclin E facilitates dysplastic hepatocytes to bypass G1/S checkpoint in hepatocarcinogenesis. *J Gastroenterol Hepatol* 2013; **28**: 1545-1554 [PMID: 23574010 DOI: 10.1111/jgh.12216]
- 47 Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res* 2014; **42**: D68-D73 [PMID: 24275495 DOI: 10.1093/nar/gkt1181]
- 48 Huang DW, Sherman BT, Tan Q, Kir J, Liu D, Bryant D, Guo Y, Stephens R, Baseler MW, Lane HC, Lempicki RA. DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. *Nucleic Acids Res* 2007; **35**: W169-W175 [PMID: 17576678 DOI: 10.1093/nar/gkm415]
- 49 Pettinelli P, Del Pozo T, Araya J, Rodrigo R, Araya AV, Smok G, Csendes A, Gutierrez L, Rojas J, Korn O, Maluenda F, Diaz JC, Rencoret G, Braghetto I, Castillo J, Ponichak J, Videla LA. Enhancement in liver SREBP-1c/PPAR-alpha ratio and steatosis in obese patients: correlations with insulin resistance and n-3 long-chain polyunsaturated fatty acid depletion. *Biochim Biophys Acta* 2009; **1792**: 1080-1086 [PMID: 19733654 DOI: 10.1016/j.bbdis.2009.08.015]
- 50 Janssen AW, Betzel B, Stoopen G, Berends FJ, Janssen IM, Peijnenburg AA, Kersten S. The impact of PPAR $\alpha$  activation on whole genome gene expression in human precision cut liver slices. *BMC Genomics* 2015; **16**: 760 [PMID: 26449539 DOI: 10.1186/s12864-015-1969-3]
- 51 Lee K, Kerner J, Hoppel CL. Mitochondrial carnitine palmitoyltransferase 1a (CPT1a) is part of an outer membrane fatty acid transfer complex. *J Biol Chem* 2011; **286**: 25655-25662 [PMID: 21622568 DOI: 10.1074/jbc.M111.228692]
- 52 Orellana-Gavaldà JM, Herrero L, Malandrino MI, Pañeda A, Sol Rodríguez-Peña M, Petry H, Asins G, Van Deventer S, Hegardt FG, Serra D. Molecular therapy for obesity and diabetes based on a long-term increase in hepatic fatty-acid oxidation. *Hepatology* 2011; **53**: 821-832 [PMID: 21319201 DOI: 10.1002/hep.24140]
- 53 Tan L, Narayan SB, Chen J, Meyers GD, Bennett MJ. PTC124 improves readthrough and increases enzymatic activity of the CPT1A R160X nonsense mutation. *J Inherit Metab Dis* 2011; **34**: 443-447 [PMID: 21253826 DOI: 10.1007/s10545-010-9265-5]
- 54 Kirpich I, Ghare S, Zhang J, Gobejishvili L, Kharebava G, Barve SJ, Barker D, Moghe A, McClain CJ, Barve S. Binge alcohol-induced microvesicular liver steatosis and injury are associated with down-regulation of hepatic Hdac 1, 7, 9, 10, 11 and up-regulation of Hdac 3. *Alcohol Clin Exp Res* 2012; **36**: 1578-1586 [PMID: 22375794 DOI: 10.1111/j.1530-0277.2012.01751.x]
- 55 Litherland NB, Bionaz M, Wallace RL, Looor JJ, Drackley JK. Effects of the peroxisome proliferator-activated receptor-alpha agonists clofibrate and fish oil on hepatic fatty acid metabolism in weaned dairy calves. *J Dairy Sci* 2010; **93**: 2404-2418 [PMID: 20494149 DOI: 10.3168/jds.2009-2716]
- 56 Herrmann T, Buchkremer F, Gosch I, Hall AM, Bernlohr DA, Stremmel W. Mouse fatty acid transport protein 4 (FATP4): characterization of the gene and functional assessment as a very

- long chain acyl-CoA synthetase. *Gene* 2001; **270**: 31-40 [PMID: 11404000]
- 57 **Gallardo D**, Amills M, Quintanilla R, Pena RN. Mapping and tissue mRNA expression analysis of the pig solute carrier 27A (SLC27A) multigene family. *Gene* 2013; **515**: 220-223 [PMID: 23219995 DOI: 10.1016/j.gene.2012.11.029]
- 58 **Qiu F**, Xie L, Ma JE, Luo W, Zhang L, Chao Z, Chen S, Nie Q, Lin Z, Zhang X. Lower Expression of SLC27A1 Enhances Intramuscular Fat Deposition in Chicken via Down-Regulated Fatty Acid Oxidation Mediated by CPT1A. *Front Physiol* 2017; **8**: 449 [PMID: 28706492 DOI: 10.3389/fphys.2017.00449]
- 59 **Lenz LS**, Marx J, Chamulitrat W, Kaiser I, Gröne HJ, Liebisch G, Schmitz G, Elsing C, Straub BK, Füllekrug J, Stremmel W, Herrmann T. Adipocyte-specific inactivation of Acyl-CoA synthetase fatty acid transport protein 4 (Fatp4) in mice causes adipose hypertrophy and alterations in metabolism of complex lipids under high fat diet. *J Biol Chem* 2011; **286**: 35578-35587 [PMID: 21808061 DOI: 10.1074/jbc.M111.226530]

**P- Reviewer:** Inoue K, Laguna JC **S- Editor:** Gong ZM  
**L- Editor:** Filipodia **E- Editor:** Huang Y



## Basic Study

# Effect of *Lactobacillus rhamnosus* GG supernatant on serotonin transporter expression in rats with post-infectious irritable bowel syndrome

Ya-Nan Cao, Li-Juan Feng, Yuan-Yuan Liu, Kui Jiang, Mao-Jun Zhang, Yi-Xin Gu, Bang-Mao Wang, Jia Gao, Ze-Lan Wang, Yu-Ming Wang

Ya-Nan Cao, Yuan-Yuan Liu, Kui Jiang, Bang-Mao Wang, Jia Gao, Ze-Lan Wang, Yu-Ming Wang, Department of Gastroenterology and Hepatology, Tianjin Medical University General Hospital, Tianjin 300052, China

Li-Juan Feng, Department of Functional Division, Xingtai People's Hospital, Xingtai 054031, Hebei Province, China

Mao-Jun Zhang, Yi-Xin Gu, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China

ORCID number: Ya-Nan Cao (0000-0002-8012-3590); Li-Juan Feng (0000-0003-2123-9515); Yuan-Yuan Liu (0000-0003-3484-698X); Kui Jiang (0000-0002-5935-1409); Mao-Jun Zhang (0000-0002-2236-8768); Yi-Xin Gu (0000-0003-1483-5976); Bang-Mao Wang (0000-0001-5128-6334); Jia Gao (0000-0002-4509-5096); Ze-Lan Wang (0000-0002-7454-9894); Yu-Ming Wang (0000-0002-2018-2273).

**Author contributions:** Cao YN and Feng LJ contributed to essential research and statistical analysis; Wang YM, Jiang K, and Wang BM designed the study; Zhang MJ, Gu YX and Liu YY were mainly responsible for cultivation of bacteria; Gao J, Liu YY and Wang ZL contributed to raising animals; Cao YN and Feng LJ edited the article; Wang YM supported the financial arrangement.

**Supported by the National Natural Science Foundation of China, No. 81570489.**

**Institutional review board statement:** This study was approved by the Tianjin Medical University General Hospital.

**Institutional animal care and use committee statement:** All procedures involving animals were reviewed and approved by the Animal Ethics and Welfare Committee of Tianjin Medical University.

**Conflict-of-interest statement:** The authors declare that they

have no competing interests.

**Data sharing statement:** Readers can get the data of this paper by contacting us via E-mail: [ywang12@tmu.edu.cn](mailto:ywang12@tmu.edu.cn).

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**Manuscript source:** Unsolicited manuscript

**Correspondence to:** Yu-Ming Wang, MD, PhD, Attending Physician, Department of Gastroenterology and Hepatology, Tianjin Medical University General Hospital, No. 154, Anshan Road, Heping District, Tianjin 300052, China. [ywang12@tmu.edu.cn](mailto:ywang12@tmu.edu.cn)  
Telephone: +86-22-60362608  
Fax: +86-22-60363768

**Received:** November 9, 2017

**Peer-review started:** November 9, 2017

**First decision:** November 30, 2017

**Revised:** December 6, 2017

**Accepted:** December 12, 2017

**Article in press:** December 12, 2017

**Published online:** January 21, 2018

## Abstract

### AIM

To evaluate the effect of *Lactobacillus rhamnosus* GG supernatant (LGG-s) on the expression of serotonin transporter (SERT) in rats with post-infectious irritable



bowel syndrome (PI-IBS).

## METHODS

*Campylobacter jejuni* 81-176 ( $10^{10}$  CFU/mL) was used to induce intestinal infection to develop a PI-IBS model. After evaluation of the post-infectious phase by biochemical tests, DNA agarose gel electrophoresis, abdominal withdrawal reflex (AWR) test, and the intestinal motility test, four PI-IBS groups received different concentrations of LGG-s for 4 wk. The treatments were maintained for 1.0, 2.0, 3.0 or 4.0 wk during the experiment, and the colons and brains were removed for later use each week. SERT mRNA and protein levels were detected by real-time PCR and Western blot, respectively.

## RESULTS

The levels of SERT mRNA and protein in intestinal tissue were higher in rats treated with LGG-s than in control rats and PI-IBS rats gavaged with PBS during the whole study. Undiluted LGG-s up-regulated SERT mRNA level by 2.67 times compared with the control group by week 2, and SERT mRNA expression kept increasing later. Double-diluted LGG-s was similar to undiluted-LGG-s, resulting in high levels of SERT mRNA. Triple-diluted LGG-s up-regulated SERT mRNA expression level by 6.9-times compared with the control group, but SERT mRNA expression decreased rapidly at the end of the second week. At the first week, SERT protein levels were basically comparable in rats treated with undiluted LGG-s, double-diluted LGG-s, and triple-diluted LGG-s, which were higher than those in the control group and PBS-treated PI-IBS group. SERT protein levels in the intestine were also comparable in rats treated with undiluted LGG-s, double-diluted LGG-s, and triple-diluted LGG-s by the second and third weeks. SERT mRNA and protein levels in the brain had no statistical difference in the groups during the experiment.

## CONCLUSION

LGG-s can up-regulate SERT mRNA and protein levels in intestinal tissue but has no influence in brain tissue in rats with PI-IBS.

**Key words:** Serotonin transporter; Intestinal infection; *Lactobacillus rhamnosus* supernatant; Irritable bowel syndrome

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**Core tip:** There are few reports on the effect of the supernatant of *Lactobacillus rhamnosus* GG (LGG) on serotonin transporter (SERT) expression in rats with post-infectious irritable bowel syndrome (PI-IBS). An experimental rat model of PI-IBS was developed by *Campylobacter jejuni* infection. SERT levels in intestinal and brain tissues were detected to evaluate the effect of LGG-s.

Cao YN, Feng LJ, Liu YY, Jiang K, Zhang MJ, Gu YX, Wang BM, Gao J, Wang ZL, Wang YM. Effect of *Lactobacillus rhamnosus* GG supernatant on serotonin transporter expression in rats with post-infectious irritable bowel syndrome. *World J Gastroenterol* 2018; 24(3): 338-350 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i3/338.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i3.338>

## INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder (FGD), annually affecting 12% to 30% of the population worldwide<sup>[1-5]</sup>. IBS can be divided into four subtypes, namely, IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), mixed-type IBS (IBS-M), and untyped IBS (IBS-U)<sup>[6-8]</sup>. Acute infectious gastroenteritis (IGE) is an important risk factor for developing IBS, with 5% to 31% of the patients developing post-infectious IBS (PI-IBS)<sup>[9-12]</sup>. Recent reports indicate that abnormalities in serotonergic signaling systems are involved in the development of PI-IBS, particularly those affecting serotonin (5-HT) levels in the gastrointestinal tract<sup>[13-15]</sup>.

As a signal transducer and neurotransmitter, 5-HT modulates intestinal fluid secretion, gut motility, and gastrointestinal sensation<sup>[16]</sup>. Serotonin transporter (SERT) is a universally existing transmembrane transport protein that plays a key role in 5-HT reuptake<sup>[15,17]</sup>. SERT has two important polymorphic areas. The first is named 5-HT-transporter gene-linked polymorphic region (5-HTTLPR), which is located in the regulatory region of its gene (SLC6A4; chromosome 17q11.1-q12)<sup>[18]</sup>. The most frequently studied variant is subdivided into long (L) and short (S) alleles<sup>[19,20]</sup>. The transcriptional efficiency of the L/L genotype is significantly higher than that of the L/S and S/S genotypes<sup>[21]</sup>. Furthermore, the frequency of the L/L genotype in C-IBS was significantly higher than that in D-IBS, and the S/S genotype is higher in D-IBS<sup>[21-24]</sup>. In other words, the expression of SERT is higher in C-IBS, and lower in D-IBS. The second is named STin2, which is also called variable number tandem repeats (VNTR). Wang *et al.*<sup>[25]</sup> have found a higher ratio of STin2.12/10 and a lower ratio of STin2.12/12 in IBS patients, and there were no significant differences between different subtypes. However, a small-scale study on SERT in PI-IBS, conducted by Wheatcroft *et al.*<sup>[26]</sup>, showed that SERT expression was reduced in PI-IBS patients.

*Lactobacillus rhamnosus* GG (LGG) is the best studied member of the lactic acid bacteria and is known to have positive effects on human health<sup>[27]</sup>. Probiotics such as LGG are potential treatment options in patients with IBS<sup>[28]</sup>. LGG could exclude pathogens, promote mucosal immunity against *Salmonella* infection<sup>[29]</sup>, and reduce the rotavirus-related diarrhea by increasing the levels of interferon- $\gamma$  (IFN- $\gamma$ )<sup>[30]</sup>. The ESPGHAN Working Group recommends using LGG for preventing

nosocomial diarrhea<sup>[31]</sup>. Furthermore, LGG reduces the frequency and severity of abdominal pain in children with IBS<sup>[32]</sup>, along with improving disease severity, especially in IBS-D and IBS-A subtypes<sup>[33]</sup>.

Our previous study had confirmed that *Lactobacillus rhamnosus* GG supernatant (LGG-s) could up-regulate SERT mRNA and protein levels in intestinal epithelial cells and mouse intestinal tissues<sup>[34]</sup>. The aim of this study was to investigate the effects of LGG-s on the expression of SERT mRNA and SERT protein (SERT-P) in the colon and brain in a rat model of PI-IBS.

## MATERIALS AND METHODS

### Bacterial culture, LGG-s, and *Campylobacter jejuni*

LGG [53103, American Type Culture Collection (ATCC), United States] was incubated in *Lactobacillus* MRS broth (Oxoid CM0359) at 37 °C for 24 h, according to ATCC guidelines, diluted in MRS broth, and cultured again at 37 °C to reach log phase with the density determined as 0.5 at A<sub>600</sub><sup>[34]</sup>. The culture suspension was centrifuged at 4000 *g* for 15 min, then the supernatant was collected and filtered through 0.20-μm filters<sup>[35]</sup>.

*C. jejuni* 81-176 (BAA-2151, ATCC, United States) was grown on Skirrow's selective medium (Columbia Agar Base, Oxoid CM0331, supplemented with 5% sheep blood and *Campylobacter* selective supplement, Oxoid SR0117) at 42 °C under micro-aerobic conditions for 24 h. The bacterial colony was obtained with an inoculating loop and diluted in phosphate buffered solution (PBS), until the concentration reached 1.0 × 10<sup>10</sup> CFU/mL. The preliminary experiments showed that the concentration of 10<sup>10</sup> CFU/mL achieved a higher diarrhea rate, visceral hypersensitivity, and serious clinical symptoms. Bacterial concentrations were measured with a spectrophotometer (TECAN infinite M200 PRO, Switzerland)<sup>[36]</sup>.

### Animal studies

The study was performed on male Sprague-Dawley rats (aged between 7 and 8 wk) obtained from Laboratory Animal Center of Chinese People's Liberation Army General Hospital (Beijing, China). The rats were kept under the following conditions: the temperature of 23 °C ± 1 °C, a 12h/12 h light/dark cycle (lighting from 08:00 to 20:00), and free access to a sterile diet. The rats were then divided into two groups. The first group of rats were designated as the control group (*n* = 25, normal and healthy), which was given PBS (2 mL/d per rat), and another group as the model group of PI-IBS (*n* = 85), which was given *C. jejuni* (10<sup>10</sup> CFU/mL, 2 mL/d per rat) through an intra-gastric needle (Thermo Fisher Scientific, Hampton, United States). The gavage continued for 7 d. Then, the stool culture, body weight of the rats, and relative content of stool water were tested to evaluate the phase of infection.

If the rats get rid of infection with a higher Bristol

score of faeces, faster intestinal transit, and visceral hypersensitivity, they were considered to enter the post-infectious phase as PI-IBS.

After the model evaluation, the rats were regrouped; the control group was designated as M (*n* = 20) and given PBS, and the PI-IBS model group was divided into four groups and given PBS, undiluted LGG-s, double diluted LGG-s, and triple diluted LGG-s, respectively (A, B, C and D, respectively, *n* = 20) through a gavage needle<sup>[34]</sup>. PBS or LGG-s was administered at 2 mL/d per rat, and the treatments were maintained for 1.0, 2.0, 3.0 or 4.0 wk during the experiment. Five rats of each group were sacrificed; the colons and brains were removed for later use each week.

During all experiments, housing and diet conditions were the same for all groups. Rats infected with *C. jejuni* were housed in another room to prevent cross-contamination from infected to uninfected. The protocol was approved by the Animal Use and Care Committee of Tianjin Medical University (Figure 1).

### *Campylobacter* gavage

Before the infection, all rats received 1 mL of 5% (w/v) bicarbonate solution *via* a ball-tipped inoculating needle to neutralize the gastric acid. Thirty minutes later, *C. jejuni* in 2 mL of PBS was given to the PI-IBS model group and 2 mL of sterile PBS to the control group<sup>[37]</sup>.

### Assessment of acute colonization by *C. jejuni*

The fresh stool specimens were cultured for the presence of *C. jejuni* on *Campylobacter* selective agar plates using biochemical tests and DNA agarose gel electrophoresis, and the general condition, body weight, and relative content of stool water were observed or tested on the 3<sup>rd</sup>, 7<sup>th</sup>, 14<sup>th</sup>, 28<sup>th</sup>, 42<sup>nd</sup>, 56<sup>th</sup>, and 70<sup>th</sup> days after gavage. Successful intestinal colonization was defined by the detection of *C. jejuni* in stool at least once, and clearance of infection was defined by two consecutive tests with negative culture.

Biochemical tests contain catalase test, oxidase test, hippurate hydrolysis test, and 3-indoylacetate hydrolysis test (GB 4789.9-2014, China). Total DNA was extracted using a *Campylobacter* Nucleic Acid Test Kit (ZC-CAMPY-003, Kangda Zhongchuang Biotechnology Co. LTD, China) and tested by agarose gel electrophoresis.

For measuring the relative content of stool water, fresh stools were obtained from rats under manual restraint by spontaneous or perianal-stimulated defecation, weighed, and then dried at 50 °C for 72 h followed by room temperature for 48 h. Dry stools were weighed again to determine the percent wet weight of stool.

### Determination of PI-IBS

In the case of two consecutive tests with negative culture, rats were considered to get rid of infection. Rats without infection were kept separately from infected rats. After all of the rats no longer had detectable *C.*

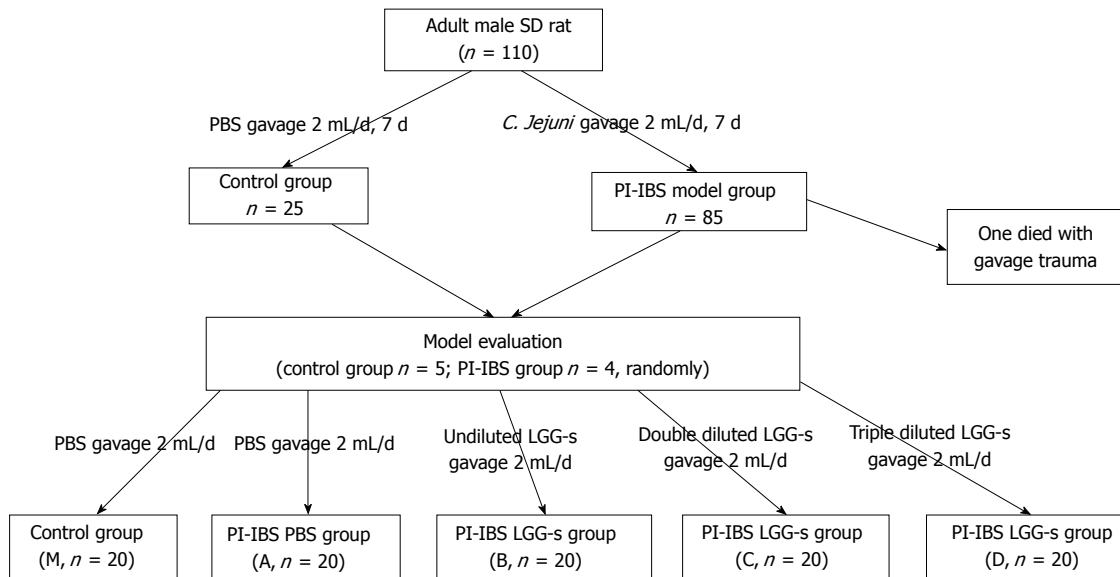


Figure 1 Experimental flow chart.

*jejuni* in the stool, they were considered to be in the post-infectious time period. Fresh stool was collected for 3 consecutive days from all rats and graded by a modified Bristol Stool score<sup>[38]</sup>. Normal stool was graded as 1, soft and poorly formed stool graded as 2, and watery stool as 3. Five rats from the control group and four from the model group were randomly chosen to perform the tests. Visceral hypersensitivity was evaluated by abdominal withdrawal reflex (AWR) test, and the intestinal motility was detected.

AWR experiment was done as previously reported<sup>[39]</sup>. Rats were fasted for 18 h before the test was performed. The night before AWR, balloons (7–8 mm diameter) were inflated overnight to stretch the latex, then the balloons became compliant. Following anesthesia by ether inhalation, a balloon coated with paraffin oil was inserted into the rectum with the tail of the balloon, 1 cm from the anus, fixed at the base of the tail. The balloon was connected *via* a double barreled cannula, with one joint connected to the air pump and another connected to the pressure gauge. Rats were given 30 min to accommodate the environment. Then, the balloon was distended at the pressures of 20, 40, 60 and 80 mmHg. Distention was sustained for 15 s, at intervals of 10 min. The distention was performed three times at each pressure, and the AWR scores were recorded. AWR was done by researchers who had no understanding of the experiment.

Intestinal motility was detected by activated carbon solution gavage. Before the experiment, rats were fasted for 24 h and then given 2 mL of 10% activated carbon solution through an inoculating needle. After 50 min, rats were sacrificed by cervical dislocation. Then, a laparotomy was performed, and the whole bowel was taken out and moistened with PBS. The bowel was freely flattened on the table. The length of the

bowel along which it contains the activated carbon and the length of the whole bowel were measured, and the ratio of these two lengths was taken as the intestinal transit rate (ITR)<sup>[40]</sup>.

#### Real-time polymerase chain reaction

To evaluate the levels of SERT mRNA after treatment with LGG-s, colon and brain samples were harvested. Rats were sacrificed by cervical dislocation, and total RNA was prepared from 50 mg of tissue from each rat with Trizol, according to the manufacturer's instructions (Life, Hilden, Germany). An iScriptcDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, United States) was used to synthesize the cDNA. The PCR were set up in a volume of 20  $\mu$ L containing 1.0  $\mu$ L cDNA, 10  $\mu$ L 2  $\times$  iQSYBR Green Supermix (Roche Applied Science), and 0.6  $\mu$ L both forward and reverse primers, replenished with DEPC treated ddH<sub>2</sub>O. Quantitative RT-PCR was performed on an ABI One plus setup PCR thermocycler. The sequences of primers are given in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as an internal control. Relative mRNA expression was calculated using the  $2^{-\Delta\Delta Ct}$  method.

#### Western blot analysis

Proteins were extracted from colonic and cerebral tissues, and protein levels were quantified using a BCA kit (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China), according to the manufacturer's instructions. The protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% nonfat milk, incubated with a primary antibody (SERT antibody: dilution 1:5000, EPR12735, Abcam Biotech

**Table 1** Primer sequences for RT-PCR

Gene	Sequence (5'-3')
rGAPDH	Forward: 5'-CCATCAACGACCCCTTCATT-3' Reverse: 5'-GACCAGCTTCCCATTTCTCAG-3'
rSERT	Forward: 5'-ACTGTTACCAAGATGCCCTG-3' Reverse: 5'-ATCTTCATTCTCATCTCCGC-3'

rGAPDH: Rat glyceraldehyde-3-phosphate dehydrogenase; rSERT: Rat serotonin transporter.

Company, Cambridge, United Kingdom;  $\beta$ -actin: dilution 1:1000, 8457S, Cell Signaling Technology, Boston, United States) overnight at 4 °C, and then incubated with a secondary antibody (dilution 1:10000, BA1054, Boster Biological Technology Co., Ltd, Wuhan, China) for 1 h at room temperature. The immunoreactive bands were visualized using an ECL Western Blotting Substrate (Solarbio Life Sciences Co., Ltd, Beijing, China).  $\beta$ -actin was used as an internal control.

### Statistical analysis

Statistical analyses were carried out using SPSS 22.0 (SPSS, Chicago, IL, United States). Quantitative data are expressed as mean  $\pm$  standard deviation. One-way analysis of variance (ANOVA) and post hoc tests (LSD and Dunnett's T3) were used to compare the values of quantitative RT-PCR. For all analyses,  $P < 0.05$  was defined as statistical significance.

## RESULTS

### Campylobacter colonization phase

A total of 110 rats were used in the study, none showed *C. jejuni* infection prior to the first gavage (as determined by stool culture), and all rats inoculated with *C. jejuni* exhibited *C. jejuni* colonization within 3 d after gavage (1 rat died from severe gavage trauma). Thus, a total of 109 rats (25 control rats and 84 PI-IBS model rats) were included before the model evaluation.

Three to fourteen days after infection, rats in the control group had good spirits, normal activity, and glossy hair. However, rats in the PI-IBS model group were decadent, indolent, and lackluster. The general conditions were returned to normal in about 42 d.

The rat weight in the model group was significantly lower than that in the control group at the 3<sup>rd</sup>, 7<sup>th</sup>, 14<sup>th</sup> and 28<sup>th</sup> days ( $P < 0.05$ ). After that, the weight in the model group was still lower than that in the control group with no statistical significance ( $P > 0.05$ ).

The relative content of water in the fresh stool in the model group was significantly higher than that of the control group at the 3<sup>rd</sup>, 7<sup>th</sup>, 14<sup>th</sup>, 28<sup>th</sup> and 70<sup>th</sup> days ( $P < 0.05$ ). At the 42<sup>th</sup> and 56<sup>th</sup> days, the model group was a little higher than the control group (Figure 2).

### Post-infectious phase

As two consecutive tests resulted in negative culture,

rats were considered to be rid of infection. At the 42<sup>th</sup> day, 95% of the *C. jejuni* rats were no longer infected. At the 56<sup>th</sup> and 70<sup>th</sup> days, all *C. jejuni* rats tested negatively. At the 70<sup>th</sup> day, tests were done to evaluate the PI-IBS. The 3-d average Bristol score of stool and the intestinal transit rate were higher in the model group than in the control group ( $P < 0.05$ ). AWR score of rats in the model group was also higher than that of the control group. However, there was no statistical difference between the two groups at the pressures of 20 and 80 mmHg ( $P = 0.12$  and  $0.06$ , respectively) (Figure 3).

### Effects of LGG-s on SERT mRNA and SERT-P expression in rat intestinal tissues

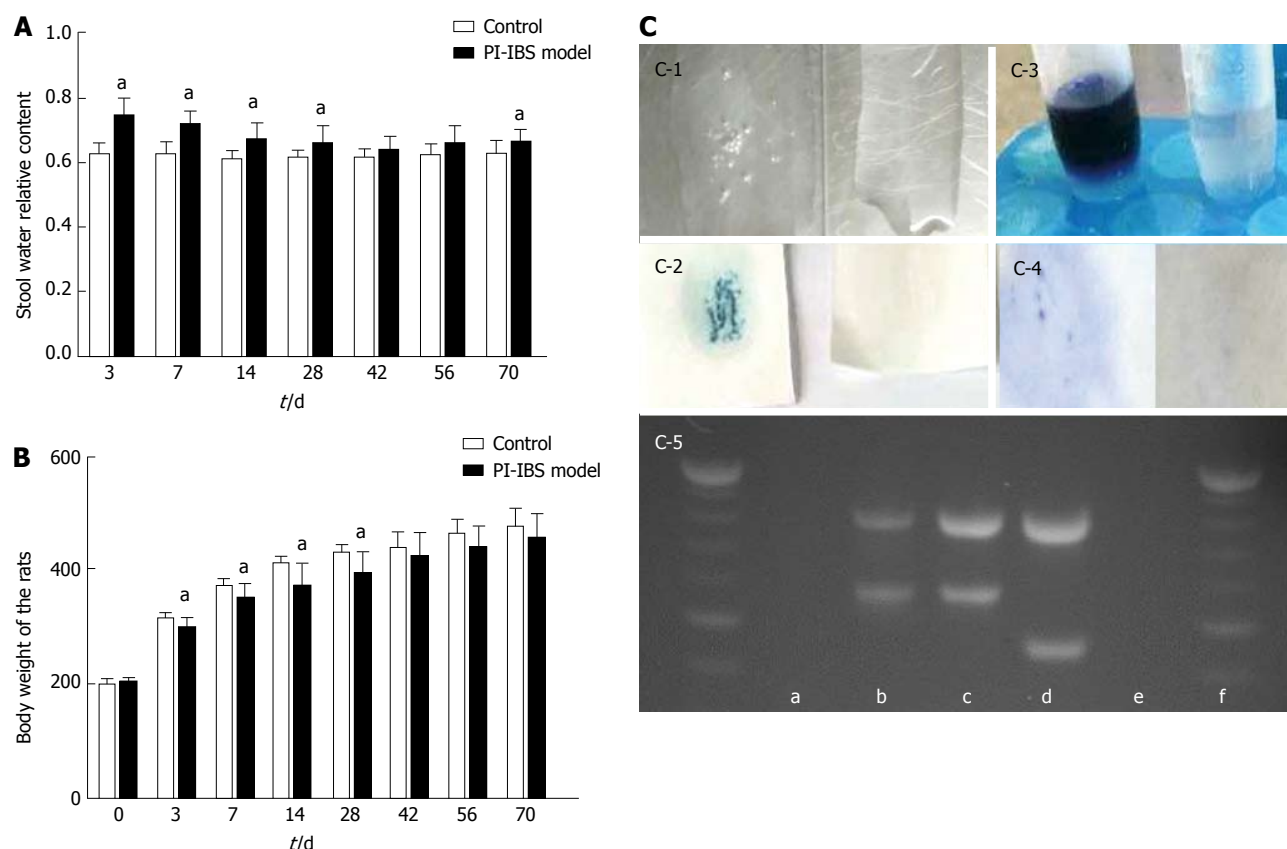
For the PI-IBS PBS gavage group (A), the levels of SERT mRNA expression were lower than those of the control group (M) throughout the experiment. However, only at the 3<sup>rd</sup> week, SERT mRNA expression in group A was significantly lower than that in group M by 0.7-fold ( $P = 0.023$ ,  $P < 0.05$ ).

Undiluted LGG-s (B) up-regulated SERT mRNA expression level by 1.8-fold compared with group A by the 1<sup>st</sup> week ( $P = 0.002$ ), and the level was slightly higher than that of group M ( $P > 0.05$ ). By the end of the 2<sup>nd</sup> week, the level of SERT mRNA in group B was 2.67 times and 3.7 times higher than those in groups M and A, respectively ( $P = 0.018$  and  $0.012$ , respectively). SERT mRNA expression in group B increased continuously, and at the 3<sup>rd</sup> week, it was 4.4-fold, 6.1-fold, and 1.7-fold more than those in groups M, A, and D (triple diluted LGG-s), respectively ( $P = 0.003$ ,  $0.003$  and  $0.028$ , respectively), although there was no significant difference between groups B and C (double diluted LGG-s). By the last week, the level of SERT mRNA in group B was only 2.1-fold higher than that in group A ( $P = 0.016$ ).

Double diluted LGG-s (C) moderately increased SERT mRNA levels by 1.8-fold compared with group A by the 1<sup>st</sup> week ( $P = 0.027$ ). The level of SERT mRNA in group C was 2.3-fold higher than that of group A at the 2<sup>nd</sup> week ( $P = 0.012$ ), and 4.6- and 6.4-fold at the 3<sup>rd</sup> week, and 1.9- and 2.6-fold at the last week compared to groups M and A ( $P = 0.012$ ,  $0.009$ ,  $0.032$ , and  $0.011$ , respectively). However, there was no statistical significance between groups C and D at the 2<sup>nd</sup> week ( $P > 0.05$ ).

Triple diluted LGG-s (D) significantly up-regulated SERT mRNA expression levels by 6.9-, 9.4-, 5.3- and 5.1-fold compared to groups M, A, B, and C at the end of the first week ( $P = 0.02$ ,  $0.01$ ,  $0.002$ , and  $0.002$ , respectively), but the level decreased rapidly at the end of the 2<sup>nd</sup> week. The SERT expression levels were similar between the 2<sup>nd</sup> week and the 3<sup>rd</sup> week, which were 2.3- and 2.5-fold higher compared to group M ( $P = 0.015$  and  $0.001$ , respectively) and 3.1- and 3.5-fold higher compared to group A ( $P = 0.009$  and  $0.028$ , respectively).





**Figure 2** Assessment of *Campylobacter* colonization phase. A: Relative content of stool water during the observation period, <sup>a</sup> $P < 0.05$  vs control group; B: Body weight of rats during the observation period, <sup>a</sup> $P < 0.05$  vs control group; C: C-1, catalase test; C-2, 3-indoylacetate hydrolysis test; C-3, hippurate hydrolysis test; C-4, oxidase test; C-5, DNA agarose gel electrophoresis (a: negative sample; b: positive sample; c: positive quality control of *Campylobacter jejuni*; d: positive quality control of *Campylobacter coli*; e: negative quality control; f: DNA ladder. Control group,  $n = 25$ ; PI-IBS group,  $n = 84$ ).

The variation tendency of SERT-P level was similar to that of SERT mRNA. SERT-P level was lower in group A than in group M during the whole experiment, and there was no significant difference only at the 3<sup>rd</sup> week ( $P = 0.177$ ). SERT-P level was similar between groups B and A, although it was lower in group B than in all other groups, at the 1<sup>st</sup> week. SERT-P level was comparable in groups B, C, and D by the 2<sup>nd</sup> and 3<sup>rd</sup> weeks. SERT-P level was slightly higher in groups B, C, and D than in group M at the 4<sup>th</sup> week, but there was no statistical significance (Figure 4).

#### Effect of LGG-s on SERT mRNA and SERT-P expression in rat brain tissues

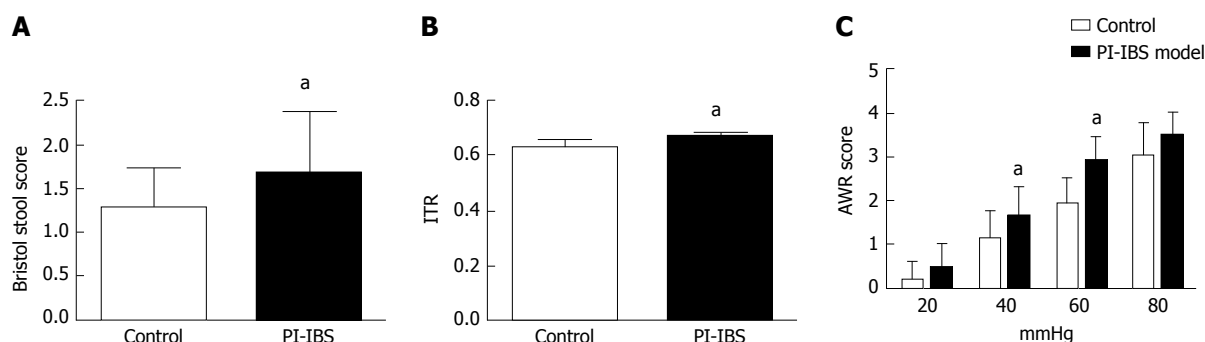
There were no statistical differences in SERT mRNA or SERT-P expression between the different dilution concentrations. The levels of SERT mRNA in groups B, C, and D had a little increase compared to groups M and A at the 1<sup>st</sup> week, but turned lower at the last week. Double diluted LGG-s resulted in a minuscule increase in SERT-P at the 3<sup>rd</sup> week, which declined at the end of the study (Figure 5).

## DISCUSSION

According to the Rome IV criteria, IBS is one of the lower

gastrointestinal tract disorders, which identify about two thirds of suffers, along with requiring that the pain either be relieved by defecation or associated with changes in stool frequency or/and consistency<sup>[8,41,42]</sup>. PI-IBS often exhibits characteristics of diarrhea, and patients often have a history of acute gastrointestinal infection<sup>[9-12]</sup>. Many studies showed that infection with *C. jejuni* strain correlates with the development of PI-IBS<sup>[43-45]</sup>. *C. jejuni* produces a range of toxins including cytolethal distending toxin, which first produces a secretory diarrhea in the small intestine, subsequently invading the distal ileum and colon to produce an inflammatory ileocolitis<sup>[46]</sup>. Therefore, *C. jejuni* was used to build a model of PI-IBS in this study. The mechanisms that underlie chronic disturbance of gut function are thought to involve chronic microscopic mucosal inflammation, visceral hypersensitivity, dysregulation of gut microbiota, and abnormal neuromuscular function<sup>[47-50]</sup>.

5-HT was found in the gastrointestinal tract and central nervous system (CNS). It functions both as a neurotransmitter and as a local hormone in the peripheral vascular system in the gut<sup>[51]</sup>. About 95% of body 5-HT is found in the gastrointestinal tract, with 90% in enterochromaffin cells (EC cell) and 10% in serotonergic neurons of myenteric plexus<sup>[51,52]</sup>. As a signal transducer and neurotransmitter, 5-HT is a key



**Figure 3 Assessment of PI-IBS phase.** A: Bristol stool score; B: Intestinal transit rate (ITR). ITR = length of the activated carbon moving in the bowel (cm)/length of the whole bowel (cm); C: AWR scores at different pressures. <sup>a</sup> $P < 0.05$  vs control group. Control group,  $n = 5$ ; PI-IBS group,  $n = 4$ .

molecule, regulating visceral perception and intestinal motility<sup>[53]</sup>. 5-HT exerts its action by binding to its receptors (5-HT<sub>1</sub> to 5-HT<sub>7</sub>) present in both intrinsic and extrinsic primary afferent neurons<sup>[54]</sup>. Serotonin receptors that are known to affect gut motor functions are those belonging to the 5-HT<sub>1</sub>, 2, 3, 4 and 7 subtypes<sup>[55-57]</sup>. 5-HT receptors contract effector cells when bound by 5-HT<sub>2A</sub> and relax cells by 5-HT<sub>4</sub> and 5-HT<sub>7</sub> subtypes<sup>[58]</sup>. Neuronal 5-HT<sub>3</sub> leads to increased release of acetylcholine from cholinergic neurons<sup>[52]</sup>. The release of 5-HT acting on effector cells leads to secretory reflexes, peristaltic reflexes, and if superfluous, diarrhea, abdominal pain, or visceral hypersensitivity<sup>[59]</sup>. Clinical trials have shown an increased level of 5-HT in IBS<sup>[60-62]</sup>. Serotonin reuptake transporter (SERT) plays an irreplaceable role in 5-HT inactivation by decreasing the content of 5-HT in the synaptic cleft<sup>[63]</sup>. SERT on the cell membrane of enterocytes is vital to transport 5-HT into the cell, with 5-HT metabolized by monoamine oxidase<sup>[64]</sup>. SERT was expressed by nearly all of the intestinal epithelial cells on the surface of the lumen<sup>[65]</sup>. Biochemical abnormalities in PI-IBS patients including hyperplasia of the EC cells and depressed 5-hydroxyindole acetic acid/5-HT ratio suggested impaired SERT function<sup>[44]</sup>. Many researchers have demonstrated that IBS patients have a remarkably lower level of SERT expression in the intestine<sup>[66]</sup>. Coates *et al.*<sup>[17]</sup> first demonstrated a significantly decreased level of SERT in IBS.

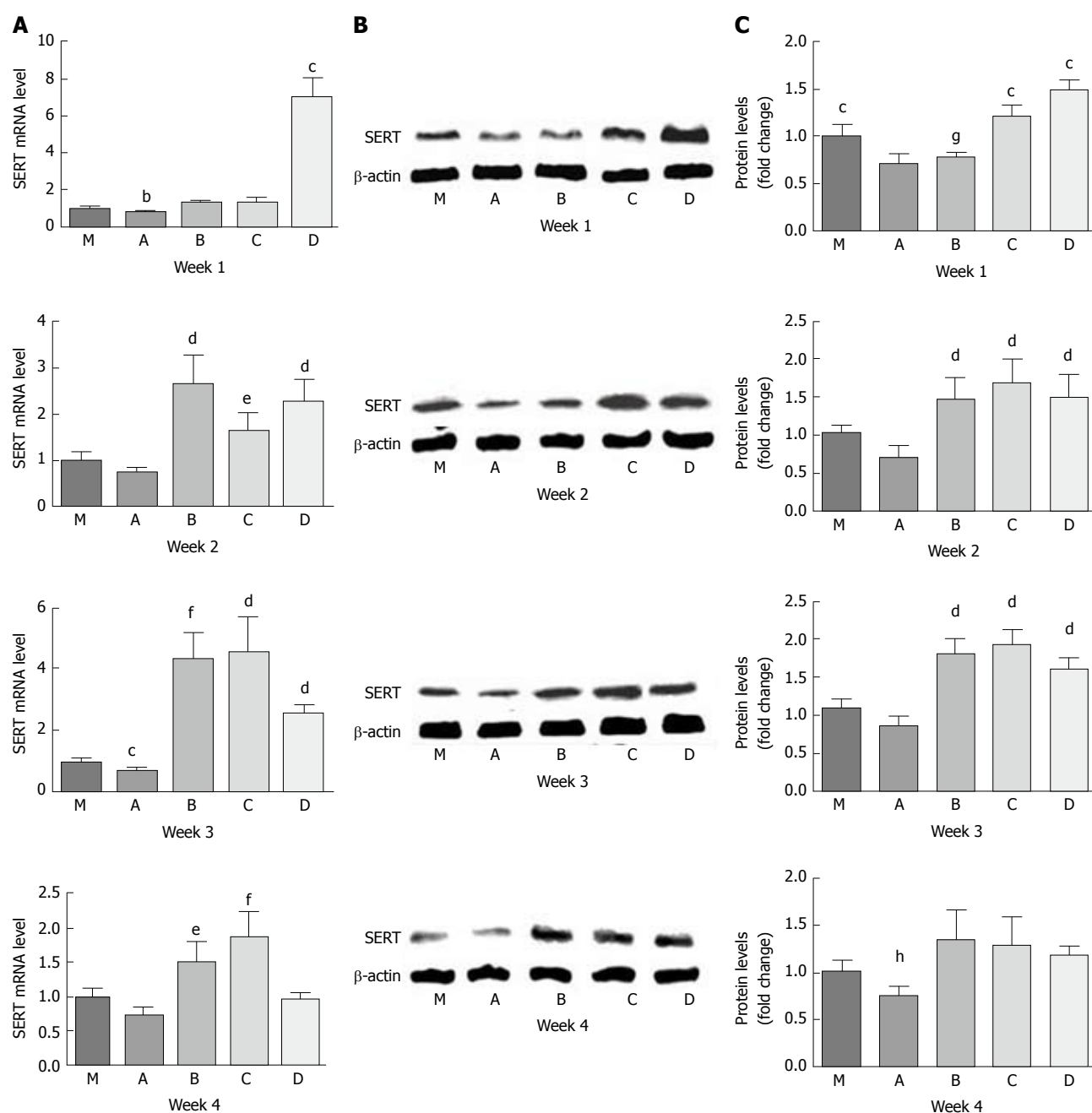
SERT expression can be regulated by a series of factors, such as gene polymorphisms, microRNAs, immunity, inflammation, gut microbiota, and growth factors<sup>[67]</sup>. The SERT gene, SLC6A4, containing 5-HTTLPR<sup>[19]</sup>, VNTR STin2<sup>[68]</sup>, and functional single nucleotide polymorphisms (SNPs), has a positive association with etiology of IBS<sup>[69]</sup>. 5-HTTLPR is the most frequently studied, which is subdivided into long (L) and short (S) alleles<sup>[19]</sup>. Previous studies found that the frequency of L/L genotype is significantly higher in C-IBS than those of L/S and S/S genotypes<sup>[21]</sup>. VNTR showed a higher ratio of STin2.12/10 and a lower ratio of STin2.12/12 in IBS<sup>[25]</sup>.

Immune activation of the gut mucosa plays a critical role in EC cell hyperplasia and reduced SERT activity in PI-IBS<sup>[70,71]</sup>. Foley *et al.*<sup>[72]</sup> found that SERT

mRNA had a lower level in D-IBS, which was correlated with increased numbers of mucosal intraepithelial lymphocytes and mast cells. Pro-inflammatory mediators, such as IFN- $\gamma$  and tumor necrosis factor- $\alpha$ , reduce the expression of SERT mRNA, SERT-P, and SERT function in Caco-2 cells<sup>[72]</sup>. However, a protective cytokine, transforming growth factor- $\beta$ 1, could rapidly activate SERT activity and inhibit intestinal inflammation *via* PI3K and syntaxin 3<sup>[73]</sup>. The acute infection of *C. jejuni* produces diarrhea in the small intestine, and leads to the inflammatory response, which increases the ratio of pro-/anti-inflammatory factors. In other words, the SERT mRNA and SERT-P levels in intestinal tissues were significantly decreased in the PI-IBS PBS group than in controls. The highest concentration of LGG-s did not induce the highest expression of SERT, which is inconsistent with a previous study which found a dose-dependent up-regulation of expression of SERT<sup>[34]</sup>. This may due to the differences in the contents of various substances (proteins, fatty acids, inorganic salts, *etc.*) in the supernatant, which, combined with previous *C. jejuni* infection, could lead to different immune activation.

In addition, gut host-microbial interactions are important factors in IBS. Studies have found that *Lactobacillus*, *Bifidobacterium*, *Actinobacteria*, and *Bacteroidetes* were decreased<sup>[74-76]</sup>, while *Proteobacteria*, *Firmicutes*, and *Firmicutes/Bacteroidetes* ratios were increased in fecal samples of IBS-D patients<sup>[77]</sup>. Enteropathogenic *E. coli* and *E. coli* Nissle 1917 could decrease SERT mRNA and increase 5-HT bioavailability<sup>[78,79]</sup>. Our previous study proved that LGG-s could up-regulate the SERT mRNA and SERT-P levels in enterocytes and mouse intestinal tissues<sup>[34]</sup>. In this study, we also proved that LGG-s had a positive effect on SERT expression in colon tissues in PI-IBS rats. It may provide a novel solution for the treatment of IBS. Further studies are needed to find whether LGG-s has any impact on the composition of rat gut microbiota.

Growth factors, such as epidermal growth factor, basic fibroblast growth factor, and nerve growth factor, may be involved in the up-regulation of SERT expression<sup>[80-82]</sup>. A protein, known as p40, expressed



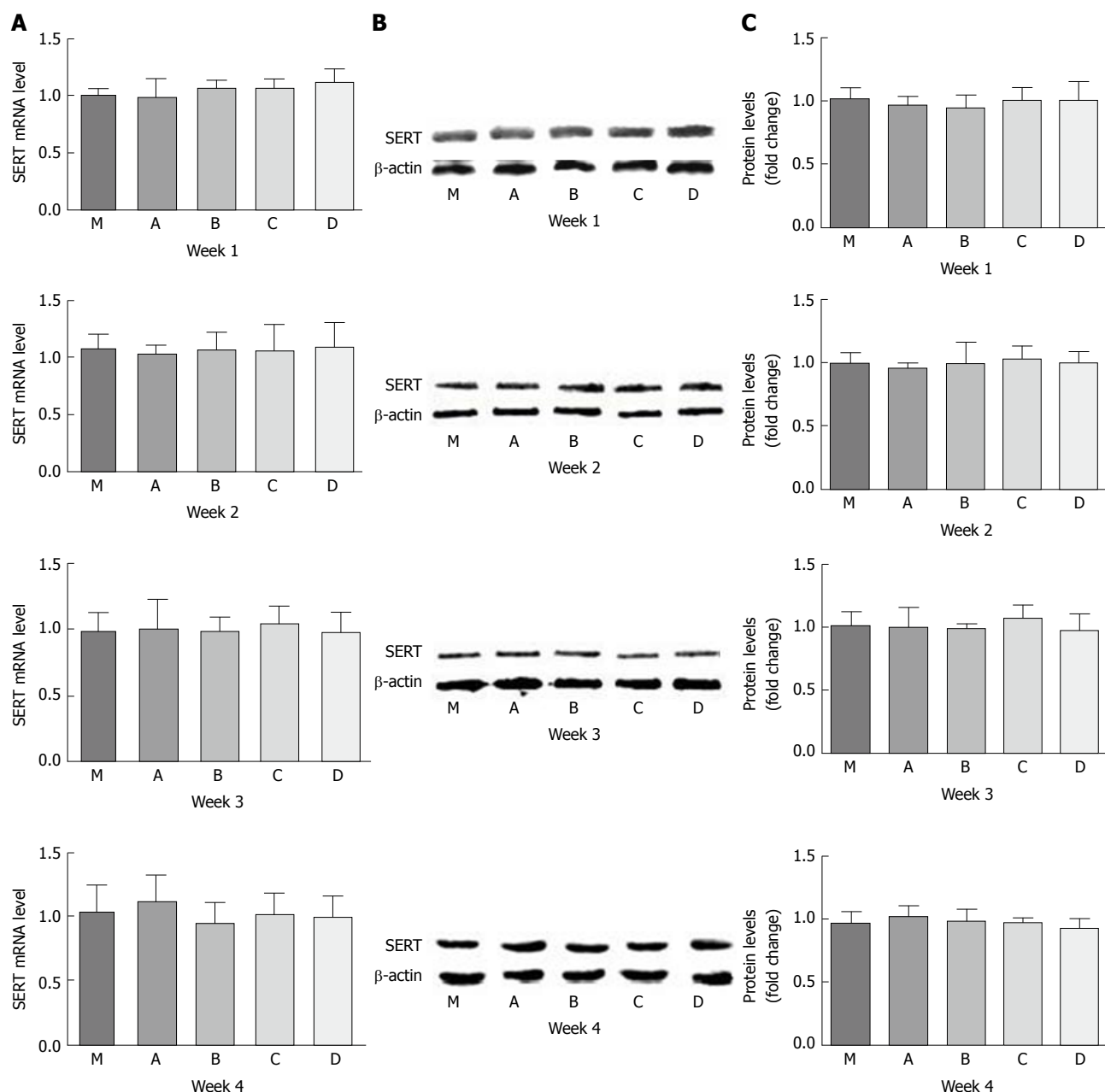
**Figure 4** Effect of LGG-s on SERT mRNA and SERT-P expression in rat intestinal tissues. A: SERT mRNA levels at the first, second, third, and fourth weeks; B: SERT-P levels at the first, second, third, and fourth weeks analyzed by Western blot; C: Quantitative analysis of SERT-P levels at the first, second, third, and fourth weeks analyzed by Western blot. <sup>b</sup> $P < 0.05$  vs B or C; <sup>c</sup> $P < 0.05$  vs all others; <sup>d</sup> $P < 0.05$  vs M or A; <sup>e</sup> $P < 0.05$  vs A; <sup>f</sup> $P < 0.05$  vs M, A or D; <sup>g</sup> $P < 0.05$  vs M, C or D; <sup>h</sup> $P < 0.05$  vs M or D. Control group,  $n = 5$ ; PI-IBS group,  $n = 5$ , each week.

by LGG, activates epidermal growth factor receptor (EGFR)<sup>[35]</sup>, which might activate the mechanism of LGG-s induced up-regulation of SERT expression.

Gender may be another factor. Although IBS is an universally disease, women are more likely to suffer from this illness than men<sup>[83]</sup>. It has been found that SERT mRNA levels in the rectal mucosa of women with IBS-D were higher than those in men. Female SERT knockout rats showed remarkable visceral hypersensitivity than male rats<sup>[84]</sup>. However, male animals were also

commonly used in PI-IBS research<sup>[85,86]</sup>. Ibeakanma *et al.*<sup>[87]</sup> found that brain-gut interactions could exaggerate peripheral nociceptive signaling in male mice with PI-IBS. An experimental model of male mouse induced by stress or *Giardia* also showed prominent visceral hypersensitivity<sup>[88]</sup>. As for this study, male rats were selected to perform the research.

Alterations in the bidirectional interactions between the gut and the nervous system play an important role in IBS pathophysiology and symptom generation.



**Figure 5** Effect of LGG-s on SERT mRNA and SERT-P expression in rat brain tissues. A: SERT mRNA levels at the first, second, third, and fourth weeks; B: SERT-P levels at the first, second, third, and fourth weeks analyzed by Western blot; C: Quantitative analysis of SERT-P levels at the first, second, third, and fourth weeks analyzed by Western blot. Control group,  $n = 5$ ; PI-IBS group,  $n = 5$ , each week.

Communication between the gut and the CNS, both in the ascending (gut-to-brain) and descending (brain-to-gut) directions, is called the gut-brain axis<sup>[89]</sup>. Gut microbes may communicate with the gut-brain axis *via* production of neuro-active and neuroendocrine molecules such as serotonin, aminobutyric acid (GABA), histamine, noradrenaline, and adrenaline<sup>[90]</sup>. *Lactobacilli* can convert glutamate into GABA<sup>[91]</sup>, and administration of *L. rhamnosus* JB-1 to mice altered the patterns of GABA receptors in the brain<sup>[92]</sup>. There is very little study about the changes of SERT in the CNS. However, no significant differences were found in SERT expression between the LGG-s treated group and the control group in this study. It is possible that the signal might

be prevented from entering the brain by the blood-brain barrier (BBB)<sup>[93]</sup>. The connections between cells in the BBB are tighter, which greatly limits the endothelial permeability *via* para-cellular and trans-cellular transport pathways<sup>[94]</sup>. The exchange of substances in the blood and brain is mainly accomplished by various transporters expressed in vascular endothelial cells<sup>[95]</sup>. Regulators or neurotransmitters, secreted by LGG, may lack specific transporters. Because of the BBB, it is very difficult for 5-HT in the blood to enter the CNS, whereas 5-HT in the central and peripheral nerves is a separate system with different functions. Therefore, as a reuptake transporter, SERT may also be regulated in different ways. The effect of LGG injected directly into



the brain is still need to be studied.

In conclusion, we found that LGG-s up-regulated SERT expression in intestinal tissues, but had no statistical effect in brain tissues in PI-IBS rats. By decreasing 5-HT levels, LGG-s may be a potential strategy for helping improve clinical symptoms of IBS.

## ARTICLE HIGHLIGHTS

### Research background

Probiotics have been approved to be used to relieve irritable bowel syndrome (IBS), and *Lactobacillus rhamnosus* GG (LGG) is the best studied member of lactic acid bacteria and has supportive therapeutic efficacy in IBS. However, the mechanism remains a significant challenge to researchers. This study developed a PI-IBS model to evaluate the effect of LGG supernatant on serotonin transporter expression.

### Research motivation

This study is a part of a National Natural Science Foundation of China project. On the basis of developing an experimental model of PI-IBS, this research explored the effect of LGG-s on SERT levels in intestinal and brain tissues.

### Research objectives

This study detected the expression levels of SERT mRNA and SERT-P to evaluate the effect of LGG-s in PI-IBS rats, which were infected with *C. jejuni*. LGG-s could up-regulate SERT mRNA and SERT-P levels in rat intestinal tissues but had no influence in rat brain tissues. The more detailed research on LGG-s will contribute to more accurate treatment of IBS.

### Research methods

The model group of PI-IBS ( $n = 85$ ) was given *C. jejuni* ( $10^{10}$  CFU/mL, 2 mL/d per rat) for 7 d, then the body weight of the rats and the relative content of stool water were measured to evaluate the phase of infection, and the fresh stool specimens were cultured for the presence of *C. jejuni* on *Campylobacter* selective agar plates. After the model evaluation, the rats were regrouped, and each group was gavaged with different concentrations of LGG-s. The treatments were maintained for 1.0, 2.0, 3.0 or 4.0 wk during the experiment. Then, SERT expression was detected by RT-PCR and Western blot to evaluate the effect of LGG-s.

### Research results

The levels of SERT mRNA and SERT-P in intestinal tissues were up-regulated by treatment with LGG-s of different concentrations. Triple-diluted LGG-s showed a more significant difference within a short term, while, in the long run, undiluted and double-diluted LGG-s proved better. However, there were no significant differences in SERT mRNA and SERT-P in the brain tissues between each group, with or without treatment with LGG-s. Some factors and differences in the contents of various substances (proteins, fatty acids, inorganic salts, etc.) in the supernatant may induce a different increase in SERT levels. More detailed research about LGG-s is needed.

### Research conclusions

This study demonstrates that LGG-s up-regulates SERT expression in intestinal tissues, but has no statistical effect in brain tissues in PI-IBS rats. The previous study has proved that LGG-s could up-regulate the SERT levels in intestinal tissues in healthy mice. Moreover, LGG-s led to dose-dependent expression of SERT. The contents of substances in the supernatant, combined with their different concentrations, molecular mass, and previous *C. jejuni* infection, may result in this phenomenon. Therefore, more detailed research about LGG-s and relief of clinical symptoms with the treatment of LGG-s would be done in our next work.

### Research perspectives

The infection with *C. jejuni* could help to build a PI-IBS model with lower expression of SERT. For the future accurate treatment of IBS, proteomics

analysis of LGG-s is important and urgent.

## ACKNOWLEDGMENTS

We thank our laboratory counselors, Jing-Wen Zhao and Wei-Qiang Wang, for their valuable support and guidance in the research work.

## REFERENCES

- 1 **Drossman DA**, Hasler WL. Rome IV-Functional GI Disorders: Disorders of Gut-Brain Interaction. *Gastroenterology* 2016; **150**: 1257-1261 [PMID: 27147121 DOI: 10.1053/j.gastro.2016.03.035]
- 2 **Kim DY**, Camilleri M. Serotonin: a mediator of the brain-gut connection. *Am J Gastroenterol* 2000; **95**: 2698-2709 [PMID: 11051338 DOI: 10.1111/j.1572-0241.2000.03177.x]
- 3 **Drossman DA**, Camilleri M, Mayer EA, Whitehead WE. AGA technical review on irritable bowel syndrome. *Gastroenterology* 2002; **123**: 2108-2131 [PMID: 12454866 DOI: 10.1053/gast.2002.37095]
- 4 **Porter CK**, Faix DJ, Shiau D, Espiritu J, Espinosa BJ, Riddle MS. Postinfectious gastrointestinal disorders following norovirus outbreaks. *Clin Infect Dis* 2012; **55**: 915-922 [PMID: 22715178 DOI: 10.1093/cid/cis576]
- 5 **Xiong LS**, Chen MH, Chen HX, Xu AG, Wang WA, Hu PJ. A population-based epidemiologic study of irritable bowel syndrome in South China: stratified randomized study by cluster sampling. *Aliment Pharmacol Ther* 2004; **19**: 1217-1224 [PMID: 15153175 DOI: 10.1111/j.1365-2036.2004.01939.x]
- 6 **Longstreth GF**, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* 2006; **130**: 1480-1491 [PMID: 16678561 DOI: 10.1053/j.gastro.2005.11.061]
- 7 **Engsbro AL**, Simren M, Bytzer P. Short-term stability of subtypes in the irritable bowel syndrome: prospective evaluation using the Rome III classification. *Aliment Pharmacol Ther* 2012; **35**: 350-359 [PMID: 22176384 DOI: 10.1111/j.1365-2036.2011.04948.x]
- 8 **Ford AC**, Bercik P, Morgan DG, Bolino C, Pintos-Sanchez MI, Moayyedi P. Validation of the Rome III criteria for the diagnosis of irritable bowel syndrome in secondary care. *Gastroenterology* 2013; **145**: 1262-70.e1 [PMID: 23994201 DOI: 10.1053/j.gastro.2013.08.048]
- 9 **Marshall JK**, Thabane M, Garg AX, Clark WF, Moayyedi P, Collins SM; Walkerton Health Study Investigators. Eight year prognosis of postinfectious irritable bowel syndrome following waterborne bacterial dysentery. *Gut* 2010; **59**: 605-611 [PMID: 20427395 DOI: 10.1136/gut.2009.202234]
- 10 **Marshall JK**, Thabane M, Garg AX, Clark WF, Salvadori M, Collins SM; Walkerton Health Study Investigators. Incidence and epidemiology of irritable bowel syndrome after a large waterborne outbreak of bacterial dysentery. *Gastroenterology* 2006; **131**: 445-450; quiz 660 [PMID: 16890598 DOI: 10.1053/j.gastro.2006.05.053]
- 11 **Thabane M**, Kottachchi DT, Marshall JK. Systematic review and meta-analysis: The incidence and prognosis of post-infectious irritable bowel syndrome. *Aliment Pharmacol Ther* 2007; **26**: 535-544 [PMID: 17661757 DOI: 10.1111/j.1365-2036.2007.03399.x]
- 12 **Andresen V**, Löwe B, Broicher W, Riegel B, Fraedrich K, von Wulffen M, Gappmayer K, Wegscheider K, Treszl A, Rose M, Leyer P, Lohse AW. Post-infectious irritable bowel syndrome (PI-IBS) after infection with Shiga-like toxin-producing *Escherichia coli* (STEC) O104:H4: A cohort study with prospective follow-up. *United European Gastroenterol J* 2016; **4**: 121-131 [PMID: 26966532 DOI: 10.1177/2050640615581113]
- 13 **Yan C**, Xin-Guang L, Hua-Hong W, Jun-Xia L, Yi-Xuan L. Effect of the 5-HT<sub>4</sub> receptor and serotonin transporter on visceral hypersensitivity in rats. *Braz J Med Biol Res* 2012; **45**: 948-954 [PMID: 22832600]
- 14 **Zang KH**, Shao YY, Zuo X, Rao Z, Qin HY. Oridonin Alleviates Visceral Hyperalgesia in a Rat Model of Postinflammatory Irritable Bowel Syndrome: Role of Colonic Enterochromaffin Cell and

- Serotonin Availability. *J Med Food* 2016; **19**: 586-592 [PMID: 27111743 DOI: 10.1089/jmf.2015.3595]
- 15 **Dizdar V**, Spiller R, Singh G, Hanevik K, Gilja OH, El-Salhy M, Hausken T. Relative importance of abnormalities of CCK and 5-HT (serotonin) in Giardia-induced post-infectious irritable bowel syndrome and functional dyspepsia. *Aliment Pharmacol Ther* 2010; **31**: 883-891 [PMID: 20132151]
  - 16 **Barbara G**, Cremon C. Serine proteases: new players in diarrhoea-predominant irritable bowel syndrome. *Gut* 2008; **57**: 1035-1037 [PMID: 18628370 DOI: 10.1136/gut.2008.150821]
  - 17 **Coates MD**, Mahoney CR, Linden DR, Sampson JE, Chen J, Blaszyk H, Crowell MD, Sharkey KA, Gershon MD, Mawe GM, Moses PL. Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. *Gastroenterology* 2004; **126**: 1657-1664 [PMID: 15188158]
  - 18 **Lesch KP**, Balling U, Gross J, Strauss K, Wolozin BL, Murphy DL, Riederer P. Organization of the human serotonin transporter gene. *J Neural Transm Gen Sect* 1994; **95**: 157-162 [PMID: 7865169]
  - 19 **Lesch KP**, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, Benjamin J, Müller CR, Hamer DH, Murphy DL. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science* 1996; **274**: 1527-1531 [PMID: 8929413]
  - 20 **Hu XZ**, Lipsky RH, Zhu G, Akhtar LA, Taubman J, Greenberg BD, Xu K, Arnold PD, Richter MA, Kennedy JL, Murphy DL, Goldman D. Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder. *Am J Hum Genet* 2006; **78**: 815-826 [PMID: 16642437 DOI: 10.1086/503850]
  - 21 **Wang YM**, Chang Y, Chang YY, Cheng J, Li J, Wang T, Zhang QY, Liang DC, Sun B, Wang BM. Serotonin transporter gene promoter region polymorphisms and serotonin transporter expression in the colonic mucosa of irritable bowel syndrome patients. *Neurogastroenterol Motil* 2012; **24**: 560-565, e254-e255 [PMID: 22435794 DOI: 10.1111/j.1365-2982.2012.01902.x]
  - 22 **Choi YJ**, Hwang SW, Kim N, Park JH, Oh JC, Lee DH. Association Between SLC6A4 Serotonin Transporter Gene Lanked Polymorphic Region and ADRA2A -1291C>G and Irritable Bowel Syndrome in Korea. *J Neurogastroenterol Motil* 2014; **20**: 388-399 [PMID: 24917480 DOI: 10.5056/jnm14020]
  - 23 **Zhang ZF**, Duan ZJ, Wang LX, Yang D, Zhao G, Zhang L. The serotonin transporter gene polymorphism (5-HTTLPR) and irritable bowel syndrome: a meta-analysis of 25 studies. *BMC Gastroenterol* 2014; **14**: 23 [PMID: 24512255 DOI: 10.1186/1471-230x-14-23]
  - 24 **Colucci R**, Gambaccini D, Ghisu N, Rossi G, Costa F, Tuccori M, De Bortoli N, Fornai M, Antoniolli L, Ricchiuti A, Mumolo MG, Marchi S, Blandizzi C, Bellini M. Influence of the serotonin transporter 5HTTLPR polymorphism on symptom severity in irritable bowel syndrome. *PLoS One* 2013; **8**: e54831 [PMID: 23393559 DOI: 10.1371/journal.pone.0054831]
  - 25 **Wang BM**, Wang YM, Zhang WM, Zhang QY, Liu WT, Jiang K, Zhang J. [Serotonin transporter gene polymorphism in irritable bowel syndrome]. *Zhonghua Neike Zazhi* 2004; **43**: 439-441 [PMID: 15312441]
  - 26 **Wheatcroft J**, Wakelin D, Smith A, Mahoney CR, Mawe G, Spiller R. Enterochromaffin cell hyperplasia and decreased serotonin transporter in a mouse model of postinfectious bowel dysfunction. *Neurogastroenterol Motil* 2005; **17**: 863-870 [PMID: 16336502 DOI: 10.1111/j.1365-2982.2005.00719.x]
  - 27 **Floch MH**. Recommendations for probiotic use in humans-a 2014 update. *Pharmaceuticals (Basel)* 2014; **7**: 999-1007 [PMID: 25310351 DOI: 10.3390/ph7100999]
  - 28 **McFarland LV**, Dublin S. Meta-analysis of probiotics for the treatment of irritable bowel syndrome. *World J Gastroenterol* 2008; **14**: 2650-2661 [PMID: 18461650 DOI: 10.3748/wjg.14.2650]
  - 29 **Yang GY**, Yu J, Su JH, Jiao LG, Liu X, Zhu YH. Oral Administration of Lactobacillus rhamnosus GG Ameliorates Salmonella Infantis-Induced Inflammation in a Pig Model via Activation of the IL-22BP/IL-22/STAT3 Pathway. *Front Cell Infect Microbiol* 2017; **7**: 323 [PMID: 28770173 DOI: 10.3389/fcimb.2017.00323]
  - 30 **Jiang Y**, Ye L, Cui Y, Yang G, Yang W, Wang J, Hu J, Gu W, Shi C, Huang H, Wang C. Effects of Lactobacillus rhamnosus GG on the maturation and differentiation of dendritic cells in rotavirus-infected mice. *Benef Microbes* 2017; **8**: 645-656 [PMID: 28670908 DOI: 10.3920/bm2016.0157]
  - 31 **Hojsak I**, Szajewska H, Canani RB, Guarino A, Indrio F, Kolacek S, Orel R, Shamir R, Vandenplas Y, van Goudoever JB, Weizman Z, ESPGHAN Working Group for Probiotics/Prebiotics. Probiotics for the Prevention of Nosocomial Diarrhea in Children. *J Pediatr Gastroenterol Nutr* 2018; **66**: 3-9 [PMID: 28574970 DOI: 10.1097/mpg.0000000000001637]
  - 32 **Francavilla R**, Miniello V, Magistà AM, De Canio A, Bucci N, Gagliardi F, Lionetti E, Castellaneta S, Polimeno L, Peccarisi L, Indrio F, Cavallo L. A randomized controlled trial of Lactobacillus GG in children with functional abdominal pain. *Pediatrics* 2010; **126**: e1445-e1452 [PMID: 21078735 DOI: 10.1542/peds.2010-0467]
  - 33 **Pedersen N**, Andersen NN, Végh Z, Jensen L, Ankersen DV, Felding M, Simonsen MH, Burisch J, Munkholm P. Ehealth: low FODMAP diet vs Lactobacillus rhamnosus GG in irritable bowel syndrome. *World J Gastroenterol* 2014; **20**: 16215-16226 [PMID: 25473176 DOI: 10.3748/wjg.v20.i43.16215]
  - 34 **Wang YM**, Ge XZ, Wang WQ, Wang T, Cao HL, Wang BL, Wang BM. Lactobacillus rhamnosus GG supernatant upregulates serotonin transporter expression in intestinal epithelial cells and mice intestinal tissues. *Neurogastroenterol Motil* 2015; **27**: 1239-1248 [PMID: 26088715 DOI: 10.1111/nmo.12615]
  - 35 **Yan F**, Cao H, Cover TL, Whitehead R, Washington MK, Polk DB. Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. *Gastroenterology* 2007; **132**: 562-575 [PMID: 17258729 DOI: 10.1053/j.gastro.2006.11.022]
  - 36 **Pimentel M**, Chatterjee S, Chang C, Low K, Song Y, Liu C, Morales W, Ali L, Lezcano S, Conklin J, Finegold S. A new rat model links two contemporary theories in irritable bowel syndrome. *Dig Dis Sci* 2008; **53**: 982-989 [PMID: 17934822 DOI: 10.1007/s10620-007-9977-z]
  - 37 **Jee SR**, Morales W, Low K, Chang C, Zhu A, Pokkunuri V, Chatterjee S, Soffer E, Conklin JL, Pimentel M. ICC density predicts bacterial overgrowth in a rat model of post-infectious IBS. *World J Gastroenterol* 2010; **16**: 3680-3686 [PMID: 20677340 DOI: 10.3748/wjg.v16.i29.3680]
  - 38 **Pokkunuri V**, Pimentel M, Morales W, Jee SR, Alpern J, Weitsman S, Marsh Z, Low K, Hwang L, Khoshini R, Barlow GM, Wang H, Chang C. Role of Cytolethal Distending Toxin in Altered Stool Form and Bowel Phenotypes in a Rat Model of Post-infectious Irritable Bowel Syndrome. *J Neurogastroenterol Motil* 2012; **18**: 434-442 [PMID: 23106005 DOI: 10.5056/jnm.2012.18.4.434]
  - 39 **Al-Chaer ED**, Kawasaki M, Pasricha PJ. A new model of chronic visceral hypersensitivity in adult rats induced by colon irritation during postnatal development. *Gastroenterology* 2000; **119**: 1276-1285 [PMID: 11054385]
  - 40 **Wang W**, Xin H, Fang X, Dou H, Liu F, Huang D, Han S, Fei G, Zhu L, Zha S, Zhang H, Ke M. Isomaltoligosaccharides ameliorate visceral hyperalgesia with repair damage of ileal epithelial ultrastructure in rats. *PLoS One* 2017; **12**: e0175276 [PMID: 28437458 DOI: 10.1371/journal.pone.0175276]
  - 41 **Schmulson MJ**, Drossman DA. What Is New in Rome IV. *J Neurogastroenterol Motil* 2017; **23**: 151-163 [PMID: 28274109 DOI: 10.5056/jnm16214]
  - 42 **Spiller R**. Clinical update: irritable bowel syndrome. *Lancet* 2007; **369**: 1586-1588 [PMID: 17499587 DOI: 10.1016/s0140-6736(07)60726-0]
  - 43 **Thornley JP**, Jenkins D, Neal K, Wright T, Brough J, Spiller RC. Relationship of Campylobacter toxigenicity in vitro to the development of postinfectious irritable bowel syndrome. *J Infect Dis* 2001; **184**: 606-609 [PMID: 11474430 DOI: 10.1086/322845]
  - 44 **Dunlop SP**, Jenkins D, Neal KR, Spiller RC. Relative importance of enterochromaffin cell hyperplasia, anxiety, and depression in postinfectious IBS. *Gastroenterology* 2003; **125**: 1651-1659 [PMID: 14724817]

- 45 **Swan C**, Duroudier NP, Campbell E, Zaitoun A, Hastings M, Dukes GE, Cox J, Kelly FM, Wilde J, Lennon MG, Neal KR, Whorwell PJ, Hall IP, Spiller RC. Identifying and testing candidate genetic polymorphisms in the irritable bowel syndrome (IBS): association with TNFSF15 and TNF $\alpha$ . *Gut* 2013; **62**: 985-994 [PMID: 22684480 DOI: 10.1136/gutjnl-2011-301213]
- 46 **Whitehouse CA**, Balbo PB, Pesci EC, Cottle DL, Mirabito PM, Pickett CL. *Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block. *Infect Immun* 1998; **66**: 1934-1940 [PMID: 9573072]
- 47 **Ford AC**, Talley NJ. Mucosal inflammation as a potential etiological factor in irritable bowel syndrome: a systematic review. *J Gastroenterol* 2011; **46**: 421-431 [PMID: 21331765 DOI: 10.1007/s00535-011-0379-9]
- 48 **Spiller R**, Garsed K. Postinfectious irritable bowel syndrome. *Gastroenterology* 2009; **136**: 1979-1988 [PMID: 19457422 DOI: 10.1053/j.gastro.2009.02.074]
- 49 **Saulnier DM**, Riehle K, Mistretta TA, Diaz MA, Mandal D, Raza S, Weidler EM, Qin X, Coarfa C, Milosavljevic A, Petrosino JF, Highlander S, Gibbs R, Lynch SV, Shulman RJ, Versalovic J. Gastrointestinal microbiome signatures of pediatric patients with irritable bowel syndrome. *Gastroenterology* 2011; **141**: 1782-1791 [PMID: 21741921 DOI: 10.1053/j.gastro.2011.06.072]
- 50 **Jeffery IB**, O'Toole PW, Öhman L, Claesson MJ, Deane J, Quigley EM, Simrén M. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut* 2012; **61**: 997-1006 [PMID: 22180058 DOI: 10.1136/gutjnl-2011-301501]
- 51 **Gershon MD**, Drakontides AB, Ross LL. Serotonin: synthesis and release from the myenteric plexus of the mouse intestine. *Science* 1965; **149**: 197-199 [PMID: 14305120]
- 52 **Sikander A**, Rana SV, Prasad KK. Role of serotonin in gastrointestinal motility and irritable bowel syndrome. *Clin Chim Acta* 2009; **403**: 47-55 [PMID: 19361459 DOI: 10.1016/j.cca.2009.01.028]
- 53 **Cremon C**, Carini G, Wang B, Vasina V, Cogliandro RF, De Giorgio R, Stanghellini V, Grundy D, Tonini M, De Ponti F, Corinaldesi R, Barbara G. Intestinal serotonin release, sensory neuron activation, and abdominal pain in irritable bowel syndrome. *Am J Gastroenterol* 2011; **106**: 1290-1298 [PMID: 21427712 DOI: 10.1038/ajg.2011.86]
- 54 **Barnes NM**, Sharp T. A review of central 5-HT receptors and their function. *Neuropharmacology* 1999; **38**: 1083-1152 [PMID: 10462127]
- 55 **Read NW**, Gwee KA. The importance of 5-hydroxytryptamine receptors in the gut. *Pharmacol Ther* 1994; **62**: 159-173 [PMID: 7991641]
- 56 **Galligan JJ**. Electrophysiological studies of 5-hydroxytryptamine receptors on enteric neurons. *Behav Brain Res* 1996; **73**: 199-201 [PMID: 8788502]
- 57 **Prins NH**, Briejer MR, Van Bergen PJ, Akkermans LM, Schuurkes JA. Evidence for 5-HT7 receptors mediating relaxation of human colonic circular smooth muscle. *Br J Pharmacol* 1999; **128**: 849-852 [PMID: 10556917 DOI: 10.1038/sj.bjp.0702762]
- 58 **Kuemmerle JF**, Murthy KS, Grider JR, Martin DC, Makhlof GM. Coexpression of 5-HT2A and 5-HT4 receptors coupled to distinct signaling pathways in human intestinal muscle cells. *Gastroenterology* 1995; **109**: 1791-1800 [PMID: 7498643]
- 59 **Spiller R**. Serotonin and GI clinical disorders. *Neuropharmacology* 2008; **55**: 1072-1080 [PMID: 18687345 DOI: 10.1016/j.neuropharm.2008.07.016]
- 60 **Keszthelyi D**, Troost FJ, Jonkers DM, van Eijk HM, Dekker J, Buurman WA, Masclee AA. Visceral hypersensitivity in irritable bowel syndrome: evidence for involvement of serotonin metabolism--a preliminary study. *Neurogastroenterol Motil* 2015; **27**: 1127-1137 [PMID: 26031193 DOI: 10.1111/nmo.12600]
- 61 **Zhao JM**, Lu JH, Yin XJ, Chen XK, Chen YH, Tang WJ, Jin XM, Wu LY, Bao CH, Wu HG, Shi Y. Comparison of electroacupuncture and moxibustion on brain-gut function in patients with diarrhea-predominant irritable bowel syndrome: A randomized controlled trial. *Chin J Integr Med* 2015; **21**: 855-865 [PMID: 25847778 DOI: 10.1007/s11655-015-2049-x]
- 62 **Keszthelyi D**, Troost FJ, Jonkers DM, van Eijk HM, Lindsey PJ, Dekker J, Buurman WA, Masclee AA. Serotonergic reinforcement of intestinal barrier function is impaired in irritable bowel syndrome. *Aliment Pharmacol Ther* 2014; **40**: 392-402 [PMID: 24943480 DOI: 10.1111/apt.12842]
- 63 **Bjerregaard H**, Severinsen K, Said S, Wiborg O, Sinning S. A dualistic conformational response to substrate binding in the human serotonin transporter reveals a high affinity state for serotonin. *J Biol Chem* 2015; **290**: 7747-7755 [PMID: 25614630 DOI: 10.1074/jbc.M114.573477]
- 64 **Keating C**, Beyak M, Foley S, Singh G, Marsden C, Spiller R, Grundy D. Afferent hypersensitivity in a mouse model of post-inflammatory gut dysfunction: role of altered serotonin metabolism. *J Physiol* 2008; **586**: 4517-4530 [PMID: 18653657 DOI: 10.1113/jphysiol.2008.156984]
- 65 **Chen JJ**, Li Z, Pan H, Murphy DL, Tamir H, Koepsell H, Gershon MD. Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: Abnormal intestinal motility and the expression of cation transporters. *J Neurosci* 2001; **21**: 6348-6361 [PMID: 11487658]
- 66 **Faure C**, Patey N, Gauthier C, Brooks EM, Mawe GM. Serotonin signaling is altered in irritable bowel syndrome with diarrhea but not in functional dyspepsia in pediatric age patients. *Gastroenterology* 2010; **139**: 249-258 [PMID: 20303355 DOI: 10.1053/j.gastro.2010.03.032]
- 67 **Jin DC**, Cao HL, Xu MQ, Wang SN, Wang YM, Yan F, Wang BM. Regulation of the serotonin transporter in the pathogenesis of irritable bowel syndrome. *World J Gastroenterol* 2016; **22**: 8137-8148 [PMID: 27688655 DOI: 10.3748/wjg.v22.i36.8137]
- 68 **MacKenzie A**, Quinn J. A serotonin transporter gene intron 2 polymorphic region, correlated with affective disorders, has allele-dependent differential enhancer-like properties in the mouse embryo. *Proc Natl Acad Sci USA* 1999; **96**: 15251-15255 [PMID: 10611371]
- 69 **Yuan J**, Kang C, Wang M, Wang Q, Li P, Liu H, Hou Y, Su P, Yang F, Wei Y, Yang J. Association study of serotonin transporter SLC6A4 gene with Chinese Han irritable bowel syndrome. *PLoS One* 2014; **9**: e84414 [PMID: 24392134 DOI: 10.1371/journal.pone.0084414]
- 70 **Spiller R**, Lam C. An Update on Post-infectious Irritable Bowel Syndrome: Role of Genetics, Immune Activation, Serotonin and Altered Microbiome. *J Neurogastroenterol Motil* 2012; **18**: 258-268 [PMID: 22837873 DOI: 10.5056/jnm.2012.18.3.258]
- 71 **Lee KJ**, Kim YB, Kim JH, Kwon HC, Kim DK, Cho SW. The alteration of enterochromaffin cell, mast cell, and lamina propria T lymphocyte numbers in irritable bowel syndrome and its relationship with psychological factors. *J Gastroenterol Hepatol* 2008; **23**: 1689-1694 [PMID: 19120860 DOI: 10.1111/j.1440-1746.2008.05574.x]
- 72 **Foley KF**, Pantano C, Ciolino A, Mawe GM. IFN-gamma and TNF-alpha decrease serotonin transporter function and expression in Caco2 cells. *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G779-G784 [PMID: 17170025 DOI: 10.1152/ajpgi.00470.2006]
- 73 **Nazir S**, Kumar A, Chatterjee I, Anbazhagan AN, Gujral T, Priyamvada S, Saksena S, Alrefai WA, Dudeja PK, Gill RK. Mechanisms of Intestinal Serotonin Transporter (SERT) Upregulation by TGF- $\beta$ 1 Induced Non-Smad Pathways. *PLoS One* 2015; **10**: e0120447 [PMID: 25954931 DOI: 10.1371/journal.pone.0120447]
- 74 **Simrén M**, Barbara G, Flint HJ, Spiegel BM, Spiller RC, Vanner S, Verdu EF, Whorwell PJ, Zoetendal EG; Rome Foundation Committee. Intestinal microbiota in functional bowel disorders: a Rome foundation report. *Gut* 2013; **62**: 159-176 [PMID: 22730468 DOI: 10.1136/gutjnl-2012-302167]
- 75 **Mayer EA**, Savidge T, Shulman RJ. Brain-gut microbiome interactions and functional bowel disorders. *Gastroenterology* 2014; **146**: 1500-1512 [PMID: 24583088 DOI: 10.1053/j.gastro.2014.02.037]
- 76 **Krogus-Kurikka L**, Lyra A, Malinen E, Aarnikunnas J, Tuimala J, Paulin L, Mäkituokko H, Kajander K, Palva A. Microbial community analysis reveals high level phylogenetic alterations in



- the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. *BMC Gastroenterol* 2009; **9**: 95 [PMID: 20015409 DOI: 10.1186/1471-230x-9-95]
- 77 **Malinen E**, Rinttilä T, Kajander K, Mättö J, Kassinen A, Krogius L, Saarela M, Korpela R, Palva A. Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *Am J Gastroenterol* 2005; **100**: 373-382 [PMID: 15667495 DOI: 10.1111/j.1572-0241.2005.40312.x]
  - 78 **Esmaili A**, Nazir SF, Borthakur A, Yu D, Turner JR, Saksena S, Singla A, Hecht GA, Alrefai WA, Gill RK. Enteropathogenic *Escherichia coli* infection inhibits intestinal serotonin transporter function and expression. *Gastroenterology* 2009; **137**: 2074-2083 [PMID: 19747920 DOI: 10.1053/j.gastro.2009.09.002]
  - 79 **Nzakizwanayo J**, Dedi C, Standen G, Macfarlane WM, Patel BA, Jones BV. *Escherichia coli* Nissle 1917 enhances bioavailability of serotonin in gut tissues through modulation of synthesis and clearance. *Sci Rep* 2015; **5**: 17324 [PMID: 26616662 DOI: 10.1038/srep17324]
  - 80 **Kekuda R**, Torres-Zamorano V, Leibach FH, Ganapathy V. Human serotonin transporter: regulation by the neuroprotective agent aurintricarboxylic acid and by epidermal growth factor. *J Neurochem* 1997; **68**: 1443-1450 [PMID: 9084414]
  - 81 **Kubota N**, Kiuchi Y, Nemoto M, Oyamada H, Ohno M, Funahashi H, Shioda S, Oguchi K. Regulation of serotonin transporter gene expression in human glial cells by growth factors. *Eur J Pharmacol* 2001; **417**: 69-76 [PMID: 11301061]
  - 82 **Gil C**, Najib A, Aguilera J. Serotonin transport is modulated differently by tetanus toxin and growth factors. *Neurochem Int* 2003; **42**: 535-542 [PMID: 12590935]
  - 83 **Katsumata R**, Shiotani A, Murao T, Ishii M, Fujita M, Matsumoto H, Haruma K. Gender Differences in Serotonin Signaling in Patients with Diarrhea-predominant Irritable Bowel Syndrome. *Intern Med* 2017; **56**: 993-999 [PMID: 28458330 DOI: 10.2169/internalmedicine.56.7674]
  - 84 **Galligan JJ**, Patel BA, Schneider SP, Wang H, Zhao H, Novotny M, Bian X, Kabeer R, Fried D, Swain GM. Visceral hypersensitivity in female but not in male serotonin transporter knockout rats. *Neurogastroenterol Motil* 2013; **25**: e373-e381 [PMID: 23594365 DOI: 10.1111/nmo.12133]
  - 85 **Morales W**, Pimentel M, Hwang L, Kunkel D, Pokkunuri V, Basseri B, Low K, Wang H, Conklin JL, Chang C. Acute and chronic histological changes of the small bowel secondary to *C. jejuni* infection in a rat model for post-infectious IBS. *Dig Dis Sci* 2011; **56**: 2575-2584 [PMID: 21409374 DOI: 10.1007/s10620-011-1662-6]
  - 86 **Shao YY**, Huang J, Ma YR, Han M, Ma K, Qin HY, Rao Z, Wu XA. Serum serotonin reduced the expression of hepatic transporter Mrp2 and P-gp via regulating nuclear receptor CAR in PI-IBS rats. *Can J Physiol Pharmacol* 2015; **93**: 633-639 [PMID: 26053941 DOI: 10.1139/cjpp-2015-0039]
  - 87 **Ibeakanma C**, Ochoa-Cortes F, Miranda-Morales M, McDonald T, Spreadbury I, Cenac N, Cattaruzza F, Hurlbut D, Vanner S, Bunnett N, Vergnolle N, Vanner S. Brain-gut interactions increase peripheral nociceptive signaling in mice with postinfectious irritable bowel syndrome. *Gastroenterology* 2011; **141**: 2098-2108. e5 [PMID: 21856270 DOI: 10.1053/j.gastro.2011.08.006]
  - 88 **Hsu LT**, Hung KY, Wu HW, Liu WW, She MP, Lee TC, Sun CH, Yu WH, Buret AG, Yu LC. Gut-derived cholecystokinin contributes to visceral hypersensitivity via nerve growth factor-dependent neurite outgrowth. *J Gastroenterol Hepatol* 2016; **31**: 1594-1603 [PMID: 26773283 DOI: 10.1111/jgh.13296]
  - 89 **Sharma A**, Lelic D, Brock C, Paine P, Aziz Q. New technologies to investigate the brain-gut axis. *World J Gastroenterol* 2009; **15**: 182-191 [PMID: 19132768 DOI: 10.3748/wjg.15.182]
  - 90 **Forsythe P**, Sudo N, Dinan T, Taylor VH, Bienenstock J. Mood and gut feelings. *Brain Behav Immun* 2010; **24**: 9-16 [PMID: 19481599 DOI: 10.1016/j.bbi.2009.05.058]
  - 91 **Li H**, Cao Y. Lactic acid bacterial cell factories for gamma-aminobutyric acid. *Amino Acids* 2010; **39**: 1107-1116 [PMID: 20364279 DOI: 10.1007/s00726-010-0582-7]
  - 92 **Bravo JA**, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci USA* 2011; **108**: 16050-16055 [PMID: 21876150 DOI: 10.1073/pnas.1102999108]
  - 93 **Zhao Z**, Nelson AR, Betsholtz C, Zlokovic BV. Establishment and Dysfunction of the Blood-Brain Barrier. *Cell* 2015; **163**: 1064-1078 [PMID: 26590417 DOI: 10.1016/j.cell.2015.10.067]
  - 94 **Komarova Y**, Malik AB. Regulation of endothelial permeability via paracellular and transcellular transport pathways. *Annu Rev Physiol* 2010; **72**: 463-493 [PMID: 20148685 DOI: 10.1146/annurev-physiol-021909-135833]
  - 95 **Zlokovic BV**. Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. *Nat Rev Neurosci* 2011; **12**: 723-738 [PMID: 22048062 DOI: 10.1038/nrn3114]

P- Reviewer: Touil-Boukoffa C, Yu LCH S- Editor: Gong ZM

L- Editor: Wang TQ E- Editor: Li D





## Basic Study

# Epidermal growth factor receptor-targeted immune magnetic liposomes capture circulating colorectal tumor cells efficiently

Jing-Hua Kuai, Qing Wang, Ai-Jun Zhang, Jing-Yu Zhang, Zheng-Feng Chen, Kang-Kang Wu, Xiao-Zhen Hu

Jing-Hua Kuai, Qing Wang, Ai-Jun Zhang, Jing-Yu Zhang, Zheng-Feng Chen, Kang-Kang Wu, Department of Gastroenterology, Qilu Hospital of Shandong University, Qingdao 266035, Shandong Province, China

Xiao-Zhen Hu, Department of General Surgery, Qilu Hospital of Shandong University, Qingdao 266035, Shandong Province, China

ORCID number: Jing-Hua Kuai (0000-0002-0529-0559); Qing Wang (0000-0001-8645-801X); Ai-Jun Zhang (0000-0002-1944-4352); Jing-Yu Zhang (0000-0002-8102-9870); Zheng-Feng Chen (0000-0002-3643-4340); Kang-Kang Wu (0000-0003-0175-0439); Xiao-Zhen Hu (0000-0002-7233-5286).

**Author contributions:** Kuai JH, Wang Q, Zhang AJ, Zhang JY, Chen ZF, Wu KK, and Hu XZ designed the research; Kuai JH, Wang Q, and Hu XZ performed the research; Zhang AJ and Zhang JY contributed new reagents/analytic tools; Kuai JH, Chen ZF, and Wu KK analyzed the data; and Kuai JH and Hu XZ wrote the paper.

**Institutional review board statement:** The study was reviewed and approved by the Institutional Review Board of Qilu Hospital of Shandong University.

**Conflict-of-interest statement:** All the authors declare that there is no conflict of interest to disclose.

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**Manuscript source:** Unsolicited manuscript

**Correspondence to:** Xiao-Zhen Hu, PhD, Chief Doctor,

Department of General Surgery, Qilu Hospital of Shandong University, No. 758, Hefei Road, Qingdao 266035, Shandong Province, China. [hu.xz@qilu.edu.cn](mailto:hu.xz@qilu.edu.cn)  
Telephone: +86-532-66850782  
Fax: +86-532-66850532

Received: September 8, 2017

Peer-review started: September 8, 2017

First decision: September 20, 2017

Revised: November 27, 2017

Accepted: December 4, 2017

Article in press: December 4, 2017

Published online: January 21, 2018

## Abstract

### AIM

To compare the capacity of newly developed epidermal growth factor receptor (EGFR)-targeted immune magnetic liposomes (EILs) vs epithelial cell adhesion molecule (EpCAM) immunomagnetic beads to capture colorectal circulating tumor cells (CTCs).

### METHODS

EILs were prepared using a two-step method, and the magnetic and surface characteristics were confirmed. The efficiency of capturing colorectal CTCs as well as the specificity were compared between EILs and EpCAM magnetic beads.

### RESULTS

The obtained EILs had a lipid nanoparticle structure similar to cell membrane. Improved binding with cancer cells was seen in EILs compared with the method of coupling nano/microspheres with antibody. The binding increased as the contact time extended. Compared with EpCAM immunomagnetic beads, EILs captured

more CTCs in peripheral blood from colorectal cancer patients. The captured cells showed consistency with clinical diagnosis and pathology. Mutation analysis showed same results between captured CTCs and cancer tissues.

## CONCLUSION

EGFR antibody-coated magnetic liposomes show high efficiency and specificity in capturing colorectal CTCs.

**Key words:** Epidermal growth factor receptor; Immune magnetic liposomes; Epithelial cell adhesion molecule; Circulating tumor cells; Colorectal cancer

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**Core tip:** Epidermal growth factor receptor-targeted immune magnetic liposomes (EILs) were prepared by a two-step method. The binding with capturing tumor cells (CTCs) in peripheral blood from colorectal cancer patients was compared between EILs and epithelial cell adhesion molecule (EpCAM) immunomagnetic beads. We found that EILs captured more CTCs than EpCAM immunomagnetic beads with a higher efficiency and specificity.

Kuai JH, Wang Q, Zhang AJ, Zhang JY, Chen ZF, Wu KK, Hu XZ. Epidermal growth factor receptor-targeted immune magnetic liposomes capture circulating colorectal tumor cells efficiently. *World J Gastroenterol* 2018; 24(3): 351-359 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i3/351.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i3.351>

## INTRODUCTION

Metastasis contributes most to cancer-related deaths in patients with solid tumors. Mounting evidence suggests that circulating tumor cells (CTCs), which can shed from a primary tumor mass at the earliest stages of malignant progression, play a critical role in cancer metastasis<sup>[1-3]</sup>. As a "liquid biopsy" for tumor, CTCs provide an insight into tumor biology in a critical window where intervention could actually make a difference<sup>[4-7]</sup>.

Detection and characterization of CTCs are challenging owing to their extreme scarcity in blood<sup>[8-11]</sup>. Several methods have been evolving, including immunomagnetic separation based on capture reagent-labeled magnetic beads, microfluidics-based technologies that enhance cell-surface contacts, and microfilter devices that isolate CTCs based on size difference<sup>[9,12-14]</sup>. However, the sensitivity of the aforementioned methods relies greatly on the degree of enrichment of CTCs and current techniques used are far from satisfactory<sup>[15-18]</sup>.

Clinically, the CellSearch System, approved by the United States Food and Drug Administration recently, is deemed as the standard method<sup>[19,20]</sup>. Epithelial cell

adhesion molecule (EpCAM) antibody is used to couple the magnetic beads in the system. However, EpCAM positive cells have been found in healthy persons<sup>[21]</sup>. Moreover, epithelial-mesenchymal transition (EMT), which is vital for metastasis, could result in loss of epithelial antigens in some CTCs<sup>[22-26]</sup>.

Recently, a new method, in which an EGFR antibody-coupled magnetic liposome with a bilayer membrane structure was used, was developed<sup>[27,28]</sup>. Studies showed that this method had a significant improvement in cell-capture efficiency owing to its enhanced interactions between the deformable antibody receptor-lipid bilayer structure and nanoscale cellular surface components<sup>[29,30]</sup>. Such a high-affinity cell assay can be employed to recover cancer cells from spiked whole-blood samples in a stationary magnetic separation device<sup>[31,32]</sup>. On the basis of this stationary cell-capture assay, we hypothesized that further improvement of cell-capture efficiency can be achieved by increasing the surface activity sites of magnetic beads for tumor cells.

## MATERIALS AND METHODS

### Materials

Chitosan (molecular weight,  $5 \times 10^4$ ) was supplied by Yuhuan Aoxing Biochemistry (Zhejiang, China) with a deacetylation degree of above 99%. Octadecyl quaternized carboxymethyl chitosan (OQCMC), hydrophobic magnetic nanoparticles (BM), and hydrophilic magnetic nanoparticles (LM) were all prepared in our lab. All other chemicals were of reagent grade and were used as received. The study was approved by local institutional review board and informed consent was obtained from all recruited patients.

### Preparation of EGFR-targeted immune liposomes

Modified DSPE-PEG-EGFR (1.0 mg) was weighed. After 3.0 mg of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 2.0 mg of OQCMC, and 6.0 mg of cholesterol (Chol) were dissolved in 5.0 mL of dichloromethane ( $\text{CH}_2\text{Cl}_2$ ), bare magnetic nanobeads (3 mL) were added and incubated for 10 min, followed by the addition of a certain volume of phosphate buffer solution (PBS, pH = 7.4). The echo probe was used for phacoemulsification during ice bathing. After formation of a uniform emulsion, the organic phase was removed by rotary evaporation. After magnetic separation, PBS was used for washing for three times. At this stage, immune magnetic liposomes (IMLs) were obtained.

A certain quantity of IMLs was dispersed in PBS solution. After that, EDC was added, vortexed, and incubated for 3 h. After the unreacted coupling agent was removed, EGFR-targeted immune liposomes (EILs) were obtained.

### Characterization of EILs

The particle size and distribution were determined

by quasielastic laser light scattering with a Malvern Zetasizer (Malvern Instruments, United Kingdom) at 25 °C. About 0.2 mL of EIL suspension were diluted in 2.5 mL water immediately after preparation. Each experiment was repeated three times. Zeta potential was measured using a Malvern Zetasizer (Malvern Instruments). Zeta limits ranged from -150 to 150 V. The magnetic properties of EILs were determined through using a vibrating samples magnetometer (LDJ9600-1, LDJ Electronics Inc., United States).

#### **Recognition of colorectal cancer HT-29 cells by EILs *in vitro***

HT-29 cells ( $8 \times 10^4$ ) at logarithmic phase were plated on sterile round cover glasses in a 24-well plate with 0.5 mL of culture medium containing 10% serum and incubated at 37 °C with 5% CO<sub>2</sub> overnight. When cell confluence reached > 70%, 20 µL of fluorescein (FITC)-labeled EILs were added. After culture for 30 min, the liquid was discarded and the pellet was washed with PBS three times before shaking for 2 min once. After that, 4% paraformaldehyde was added for cell fixation for 20 min, followed by washing with PBS three times again. Then, DAPI (4',6-diamidino-2-phe-nylindole) dye liquor was added in each well and incubated for 10 min away from light to stain nuclei. After washing three times with PBS, the cell membrane probe Dil was added in each well and incubated for 10 min. After washing three times with PBS, the cover glasses were removed. The fluorescence decay sealing agents were adhered on glass slides. The distributions of the immune magnetic beads inside and outside the cell membrane were observed under a laser confocal microscope.

#### **Recognition of CTCs**

A three-color immunocytochemistry method was applied to identify and enumerate CTCs from non-specifically trapped white blood cells (WBCs). The markers included PE-labeled anti-CD45 (a marker for WBCs), FITC-labeled anti-CK19 (Cytokeratin, a protein marker for epithelial cells), and DAPI for nuclear staining. Fluorescence microscopy was employed to quantify DAPI intensity and expression levels of CK19 and CD45 in individual cells. The combined information was utilized to delineate CTCs (DAPI+/CK+/CD45-, cell size > 8 µm) from WBCs (DAPI+/CK-/CD45+, size < 15 µm) and cellular debris (DAPI-).

#### **Clinical application of CTC capture and identification in colorectal cancer patients**

The CTC capture and identification methods were similar to those of CellSearch, but manually operated instead. Specifically, whole blood (7.5 mL) was collected and centrifuged at 1000 rpm/min for 10 min. After that, the upper-middle layer was transferred to a new tube and equal volume of PBS was added and mixed

evenly. Then, 120 µL of EILs were added and incubated at room temperature for 30 min. The mixture was mixed once every 10 min and the tube was inserted into the magnetic separation frame to allow absorbing for 5 min. After that, the supernatant was removed and the tube was taken out. The captured CTCs were washed once with PBS, followed by staining with 30 µL of DAPI, 30 µL of CK19-FITC, and 10 µL of CD45-PE in a mixture for 15 min. Deionized water was then used to wash twice in the magnetic separation frame. Finally, 30 µL of deionized water was added into the tube for re-suspending the cells. The blending liquid was then evenly coated in the center of a slide. After the liquid drop was dried, the cells were photographed and counted under a fluorescent microscope.

#### **DNA extraction and mutation analysis in CTCs**

The captured cells were put into a 10 µL PCR buffer with proteinase K and incubated at 60 °C overnight. After inactivating proteinase K at 95 °C for 10 min, agarose gel electrophoresis and sequencing were performed to determine mutations of the *KRAS* gene. Meanwhile, DNA extraction from peripheral blood of seven colorectal cancer patients was performed to analyze the *KRAS* mutations.

#### **Statistical analysis**

Statistical analyses were performed using Prism software (GraphPad Software, Inc., La Jolla, CA, United States). An unpaired Student's *t*-test was used to compare hydrodynamic size, diffuse efficient, and zeta potential between IMLs and EILs. A paired Student's *t*-test was used to detect differences in the number of CTCs captured by EpCAM immunomagnetic beads in comparison with EILs. A *P*-value < 0.05 was considered statistically significant.

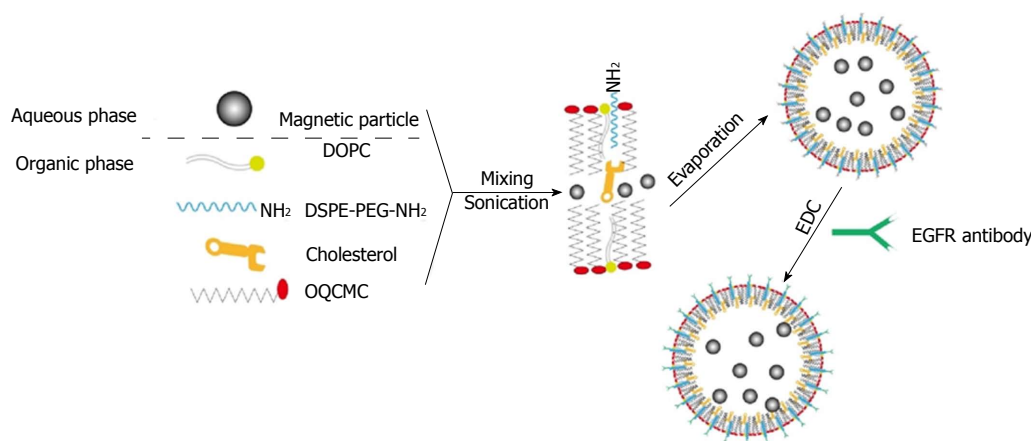
## **RESULTS**

#### **Process of preparing EILs**

A two-step method was used to prepare EILs, which was achieved through encapsulating hydrophilic or hydrophobic magnetic nanoparticles into polymeric surfactant/cholesterol vesicles. Figure 1 illustrates the preparation procedure. The as-synthesized LM can be encapsulated into the aqueous core of polymeric liposomes. Furthermore, evaporation of organic solvent can transfer BM into the aqueous phase by an interfacial process driven by the hydrophobic van der Waals interactions between the primary alkane of the stabilizing ligand in oleic acid and the secondary alkane of the OQCMC. In addition, Chol can modulate membrane fluidity, elasticity, and permeability by stabilizing the polymeric liposome system.

#### **Characterization of EILs**

Figure 2A shows the hydrodynamic diameters of



**Figure 1** Schematic diagram showing the preparation of epidermal growth factor receptor-targeted immune magnetic liposomes with hydrophilic and hydrophobic magnetic nanoparticles. EGFR: Epidermal growth factor receptor.

IMLs and EILs. The results indicated that the mean hydrodynamic size of IMLs was  $83.1 \pm 1.5$  nm, whereas the diffusion coefficient was  $1.3758 \times 10^{-14}$  m<sup>2</sup>/s. The mean hydrodynamic size of EILs was  $104.5 \pm 1.3$  nm with a diffusion coefficient of  $1.5870 \times 10^{-14}$  m<sup>2</sup>/s.

After encapsulating with OQCMC/Chol polymeric liposomes, the surface zeta potential of hydrophilic Fe<sub>3</sub>O<sub>4</sub> nanoparticles could reach +41 mv. After reaction with EGFR antibody, the surface zeta potential of IMLs could reach +37 mv. There was no significant difference in zeta potential between them (Figure 2B).

### Magnetic and surface characterization of EILs

Figure 2C shows the magnetization curves of IML and EILs at room temperature. Both samples showed a typical superparamagnetic behavior without any hysteresis loop. The saturation magnetization values of IMLs and EILs were 40 emu/g and 20 emu/g at 300 K, respectively. The volume fraction of magnetite can be further increased by increasing the number of layer of lipid coating.

### Characteristics of CTCs

As shown in Figure 3A, the captured CTCs were DAPI+/CK+/CD45-, with cell sizes larger than 8  $\mu$ m, which are consistent with the CTC criterion. In Figure 3B, the magnetic bead was stained blue by Prussian blue.

### Recognition of CTCs by EpCAM magnetic beads and EILs

Blood samples from seven colorectal cancer patients without chemotherapy were collected for CTC capture analysis. For each patient, 7.5 mL of blood was divided equally for capturing CTCs by EpCAM magnetic beads and EILs. The number of CTCs captured by EpCAM magnetic beads was 15 to 79 and that by EILs was 5 to 181. A significant difference was found between the two methods. As shown in Figure 4, the numbers of

CTCs captured by EILs in patients 1, 2, 4, 5, 6, and 7 were higher than those captured by EpCAM magnetic balls.

### Mutations analysis in CTCs

KRAS has been recognized as a marker for diagnosis and treatment of colorectal cancer. Mutations of KRAS in CTCs from the seven colorectal cancer patients were compared. Five of the seven DNA samples were successfully amplified and sequenced. We further amplified and sequenced their tumor tissue DNA, and found the results were coincident (Figure 5 and Table 1).

## DISCUSSION

In the current study, we developed new EGFR-targeted EILs for capturing colorectal CTCs. The EILs obtained showed similarity to cell membrane and could more efficiently capture colorectal CTCs compared with EpCAM immunomagnetic beads.

The higher efficiency of EILs compared to EpCAM immunomagnetic beads might be explained by the following facts. First, the obtained IMLs displayed a lipid nanoparticle structure similar to cell membrane, which can enhance contact with cancer cells<sup>[33-35]</sup>. Second, characteristics of the EILs were similar to those of IMLs (including mean hydrodynamic size, zeta potential, magnetization curves, and saturation magnetization value), which suggested that EILs could effectively bind CTC cells<sup>[30,32,36]</sup>. Third, expression of EpCAM on CTCs is dynamic<sup>[24,37]</sup>. Some cells might not express EpCAM and did not get captured using EpCAM immunomagnetic beads<sup>[22,38,39]</sup>.

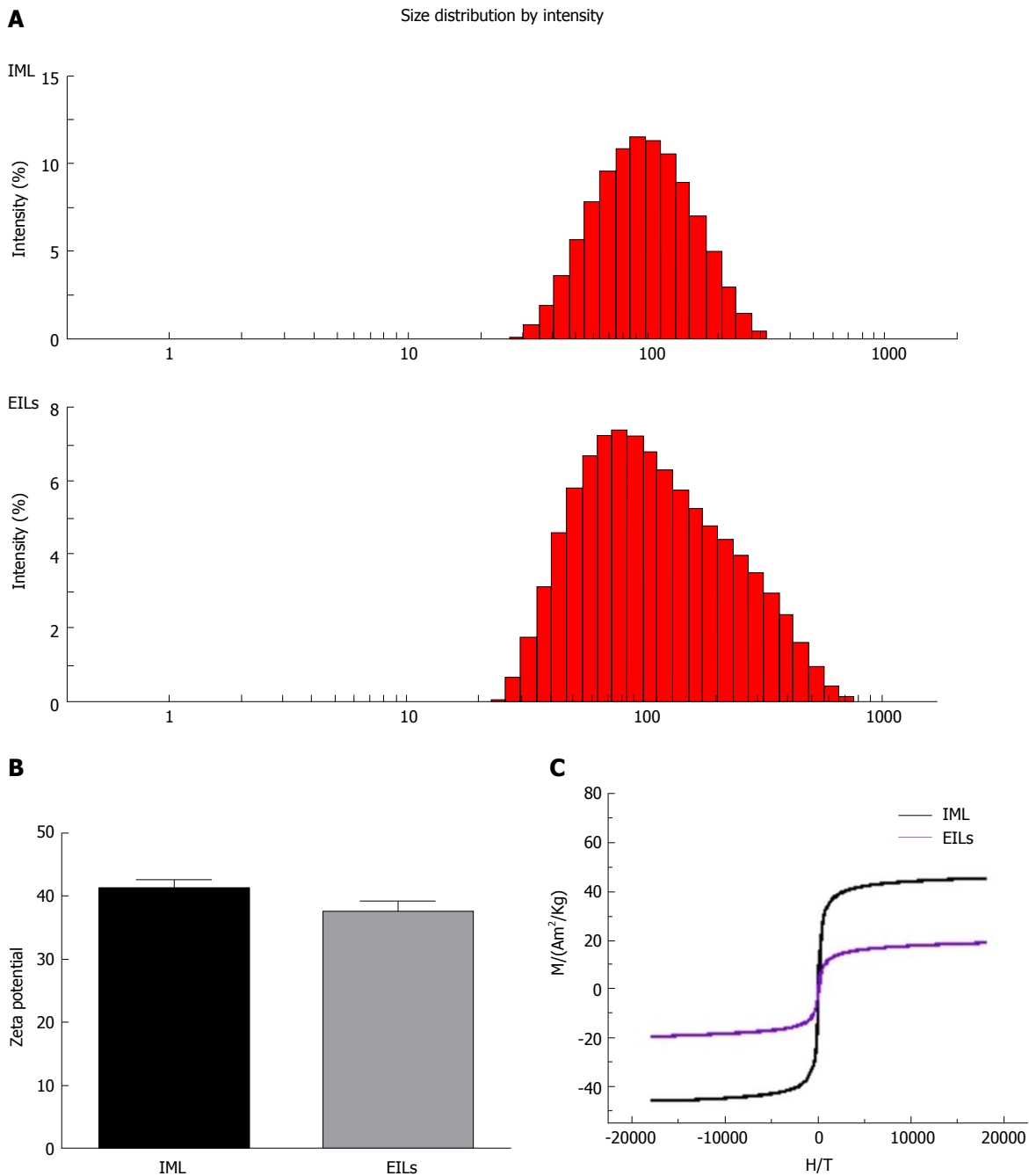
However, we should not ignore that in one patient, the number of CTCs captured by EILs was lower than that by EpCAM magnetic beads. This patient had stage I disease and the number of CTCs in the peripheral blood might be much fewer than those at advanced stages, which may be below the detection limit of EILs.



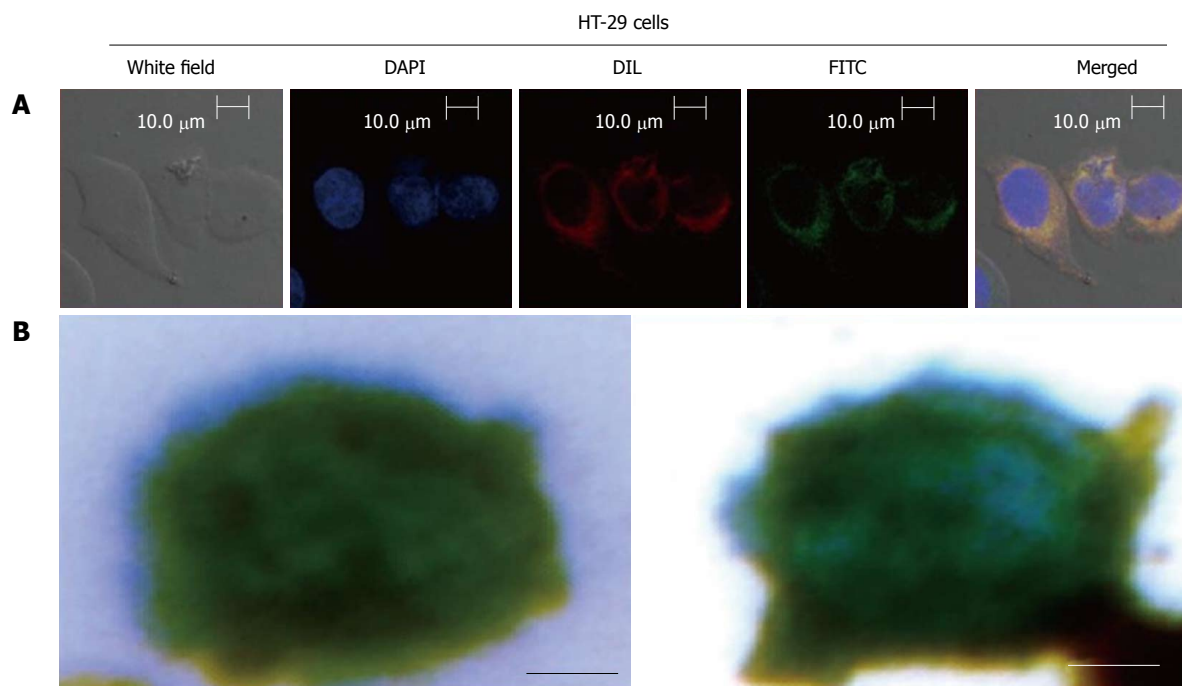
**Table 1** Comparison of gene mutations detected in DNA from circulating tumor cells and that from tissues

Sample ID	Tumor type	Clinical stage	CTC DNA		Tissue DNA	
			<i>KRAS</i> Exon 1	<i>KRAS</i> Exon 2	<i>KRAS</i> Exon 1	<i>KRAS</i> Exon 2
1	CRC	IV	WT	WT	WT	WT
2	CRC	III	WT	WT	WT	WT
3	CRC	I	NAP	NAP	WT	WT
4	CRC	IV	WT	WT	WT	WT
5	CRC	IV	WT	WT	WT	WT
6	CRC	II	NAP	NAP	WT	WT
7	CRC	III	WT	WT	WT	WT

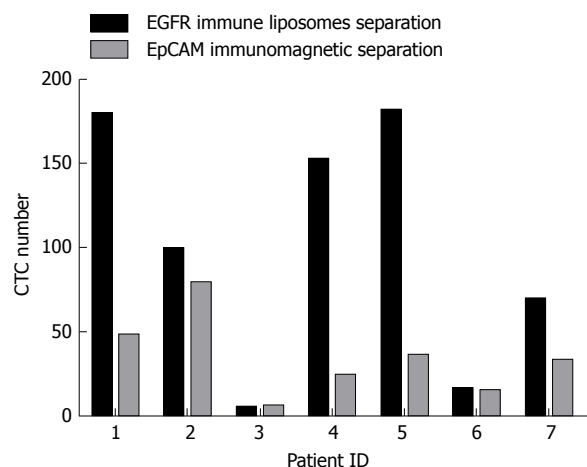
CTCs: Circulating tumor cells; CRC: Colorectal cancer; WT: Wild type; NAP: Not amplified.



**Figure 2** Characterization of epidermal growth factor receptor-targeted immune magnetic liposomes. The hydrodynamic diameter (A), zeta potential (B), and magnetization curves (C) at 300 K of magnetic polymeric liposomes are shown. IML: Immune magnetic liposomes; EGFR: Epidermal growth factor receptor; EILs: EGFR-targeted immune magnetic liposomes.



**Figure 3** Size of the captured circulating tumor cells and color of stained magnetic beads. A: Localization and distribution of EILs in HT-29 cells. Cells were cultured in FITC-labeled EILs-containing medium on bottom of a glass dish (35 mm dish with 14 mm bottom wells) for 30 min followed by treatment with DAPI dye and Dil dye for 5-10 min, separately, and subsequent examination by confocal microscopy; B: Prussian blue staining of EGFR IMLs with HT-29 Cells. (Scale bar: 5 μm). IMLs: Immune magnetic liposomes; EILs: EGFR-targeted immune magnetic liposomes.



**Figure 4** The number of circulating tumor cells captured by epithelial cell adhesion molecule magnetic beads and epidermal growth factor receptor-targeted immune magnetic liposomes. Side-by-side representation of circulating tumor cell (CTC) enumeration results obtained from our integrated CTC-capture method (normalized to 7.5 mL of blood) with EGFR immune magnetic liposomes and epithelial cell adhesion molecule (EpCAM) immunomagnetic beads on matched samples from seven patients.

Other factors such as operating mistakes might also be possible explanations. More studies with larger sample sizes are needed to validate the current findings.

The feasibility of capturing of CTCs by EILs was evaluated by mutation analysis, especially the *KRAS* gene. Five of the seven DNA samples were successfully amplified and sequenced. We found that mutations detected in CTCs were the same as those in tumor

tissues. Considering that *KRAS* was reported to be a marker for diagnosis and predicting treatment outcomes of colorectal cancer<sup>[28,40-42]</sup>, the current results suggested that detecting *KRAS* mutations in CTCs through EILs capture might be of practical use.

In 2005, Kullberg and colleagues first reported the use of magnetic liposomes modified by EGFR antibody for drug delivery to cancer cells<sup>[31]</sup>. Recently, Wang *et al.*<sup>[43]</sup> found that magnetic liposomes modified by dual antibody (the nuclear protein Ki-67 and EGFR antibody) were potentially useful in helping treat tumor cells with proliferative characteristics. Our current study further confirmed the feasibility of EILs in capturing CTCs. These findings suggested that EGFR-targeted magnetic liposomes might be of more clinical significance in the future.

There were at least two limitations in this study. First, the number of patients included in our study was small. Second, all of the colorectal cancer patients included in the study were EGFR positive, which might cause a great bias to our results as a previous study reported that the sensitivity and specificity of EGFR were lower than those of EpCAM for colorectal cancer patients<sup>[44]</sup>. Liu *et al.*<sup>[45]</sup> also reported that the positive expression rate of EGFR was only 64% (45/70). Future studies might include several specific molecular targets to improve efficiency<sup>[46]</sup>. For example, Myung *et al.*<sup>[47]</sup> successfully enhanced tumor cell isolation by a biomimetic combination of E-selectin and anti-EpCAM. Besides, combining mechanical and molecular filtration seems to be another choice to better enrich CTCs<sup>[48-51]</sup>.

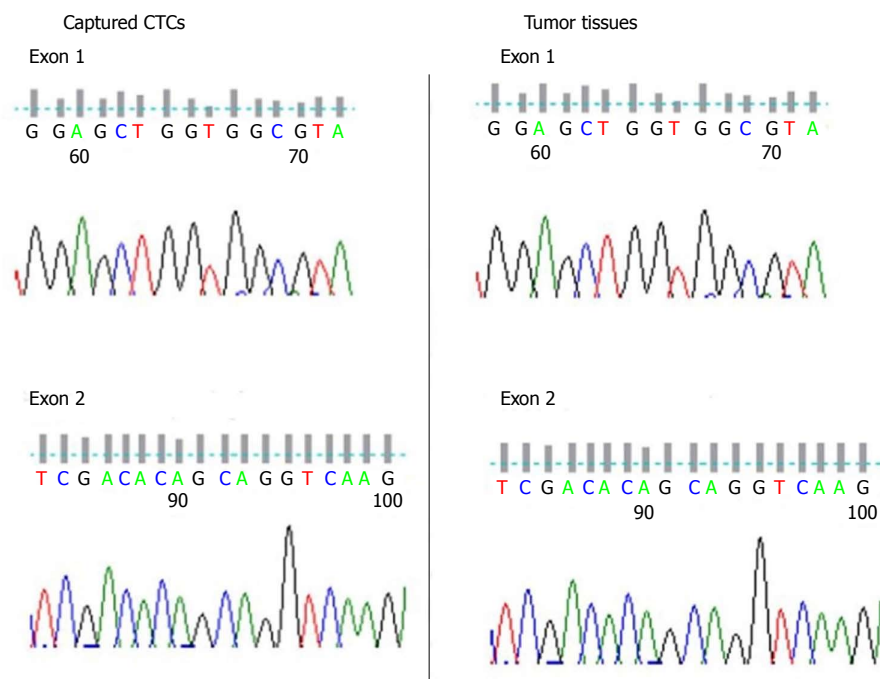


Figure 5 Gene mutation test of *KRAS* for captured circulating tumor cells and tumor tissue. CTCs: Circulating tumor cells.

In conclusion, we designed a new CTC-capture platform that combines a high-affinity cell enrichment assay based on cell capture agent (antibody)-coated nanostructured substrates and a cell membrane structure capable of improving CTC/substrate contact frequency. The synergistic effects led to better CTC capture performance in clinical blood samples compared with traditional EpCAM immunomagnetic beads. The significantly improved sensitivity of our new CTC capture technology might be useful in early detection of cancer metastasis and isolation of rare populations of cells.

## ARTICLE HIGHLIGHTS

### Research background

Metastasis contributes most to cancer-related deaths in patients with solid tumors. Mounting evidence suggests that circulating tumor cells (CTCs) which can shed from a primary tumor mass at the earliest stages of malignant progression, play a critical role in cancer metastasis. Detection and characterization of CTCs are challenging owing to their extreme scarcity in blood. However, the sensitivity of the aforementioned methods relies greatly on the degree of enrichment of CTCs and current techniques used are far from satisfactory. Recently, a new method, in which an epidermal growth factor receptor (EGFR) antibody-coupled magnetic liposome with a bilayer membrane structure was used, was developed. Studies showed that this method had a significant improvement in cell-capture efficiency owing to its enhanced interactions between the deformable antibody receptor-lipid bilayer structure and nanoscale cellular surface components. Such a high-affinity cell assay can be employed to recover cancer cells from spiked whole-blood samples in a stationary magnetic separation device.

### Research motivation

The main topics are to compare the capacity of newly developed EGFR-targeted immune magnetic liposomes (EILs) vs epithelial cell adhesion

molecule (EpCAM) immunomagnetic beads to capture colorectal circulating tumor cells (CTCs).

### Research objectives

The main objectives were to compare the capacity of newly developed EILs vs EpCAM immunomagnetic beads to CTCs. And the significantly improved sensitivity of our new CTC capture technology might be useful in early detection of cancer metastasis and isolation of rare populations of cells.

### Research methods

EILs were prepared using a two-step method, and the magnetic and surface characteristics were confirmed. The efficiency and specificity of EILs and EpCAM magnetic beads in capturing colorectal CTCs were compared. Statistical analyses were performed using Prism software (GraphPad Software, Inc., La Jolla, CA, United States). An unpaired Student's *t*-test was used to compare hydrodynamic size, diffuse efficient, and zeta potential between IMLs and EILs. A paired Student's *t*-test was used to detect differences in the number of CTCs captured by EpCAM immunomagnetic beads in comparison with EILs. A *P*-value < 0.05 was considered statistically significant.

### Research results

The obtained EILs have a lipid nanoparticle structure similar to cell membrane. Improved binding with cancer cells was seen in EILs compared with the method of coupling nano/microspheres with antibody. The binding increased as the contact time extended. Compared with EpCAM immunomagnetic beads, EILs captured more CTCs in peripheral blood from colorectal cancer patients. The captured cells showed consistency with clinical diagnosis and pathology. Mutation analysis showed same results between captured CTCs and cancer tissues.

### Research conclusions

Improved binding with cancer cells was seen in EILs compared with the method of coupling nano/microspheres with antibody. EILs were prepared using a two-step method and the magnetic and surface characteristics were confirmed. The efficiency and specificity in capturing colorectal CTCs were compared between EILs and EpCAM magnetic beads. EGFR-coated magnetic liposomes showed high efficiency and specificity in capturing colorectal CTCs. The captured cells

showed consistency with clinical diagnosis and pathology. Mutation analysis showed same results between captured CTCs and cancer tissues. The significantly improved sensitivity of our new CTC capture technology might be useful in early detection of cancer metastasis and isolation of rare populations of cells.

## Research perspectives

The significantly improved sensitivity of our new CTC capture technology might be useful in early detection of cancer metastasis and isolation of rare populations of cells.

## REFERENCES

- Pantel K, Speicher MR. The biology of circulating tumor cells. *Oncogene* 2016; **35**: 1216-1224 [PMID: 26050619 DOI: 10.1038/onc.2015.192]
- Joosse SA, Gorges TM, Pantel K. Biology, detection, and clinical implications of circulating tumor cells. *EMBO Mol Med* 2015; **7**: 1-11 [PMID: 25398926 DOI: 10.15252/emmm.201303698]
- Hardingham JE, Grover P, Winter M, Hewett PJ, Price TJ, Thierry B. Detection and Clinical Significance of Circulating Tumor Cells in Colorectal Cancer--20 Years of Progress. *Mol Med* 2015; **21** Suppl 1: S25-S31 [PMID: 26605644 DOI: 10.2119/molmed.2015.00149]
- Li N, Xiao T, Zhang Z, He R, Wen D, Cao Y, Zhang W, Chen Y. A 3D graphene oxide microchip and a Au-enwrapped silica nanocomposite-based supersandwich cytosensor toward capture and analysis of circulating tumor cells. *Nanoscale* 2015; **7**: 16354-16360 [PMID: 26391313 DOI: 10.1039/c5nr04798f]
- Rink M, Chun FK, Dahlem R, Soave A, Minner S, Hansen J, Stoupiec M, Coith C, Kluth LA, Ahyai SA, Friedrich MG, Shariat SF, Fisch M, Pantel K, Riethdorf S. Prognostic role and HER2 expression of circulating tumor cells in peripheral blood of patients prior to radical cystectomy: a prospective study. *Eur Urol* 2012; **61**: 810-817 [PMID: 22277196 DOI: 10.1016/j.eururo.2012.01.017]
- Jansson S, Bendahl PO, Larsson AM, Aaltonen KE, Rydén L. Prognostic impact of circulating tumor cell apoptosis and clusters in serial blood samples from patients with metastatic breast cancer in a prospective observational cohort. *BMC Cancer* 2016; **16**: 433 [PMID: 27390845 DOI: 10.1186/s12885-016-2406-y]
- Gazzaniga P, Raimondi C, Nicolazzo C, Carletti R, di Gioia C, Gradilone A, Cortesi E. The rationale for liquid biopsy in colorectal cancer: a focus on circulating tumor cells. *Expert Rev Mol Diagn* 2015; **15**: 925-932 [PMID: 25959553 DOI: 10.1586/14737159.2015.1045491]
- Lee GW, Kim JY, Koh EH, Kang D, Choi DS, Maeng KY, Lee JS. Plasma human mammaglobin mRNA associated with poor outcome in patients with breast cancer. *Genet Mol Res* 2012; **11**: 4034-4042 [PMID: 23212340 DOI: 10.4238/2012.November.28.2]
- Alix-Panabières C, Pantel K. Technologies for detection of circulating tumor cells: facts and vision. *Lab Chip* 2014; **14**: 57-62 [PMID: 24145967 DOI: 10.1039/c3lc50644d]
- Chen JF, Zhu Y, Lu YT, Hodara E, Hou S, Agopian VG, Tomlinson JS, Posadas EM, Tseng HR. Clinical Applications of NanoVelcro Rare-Cell Assays for Detection and Characterization of Circulating Tumor Cells. *Theranostics* 2016; **6**: 1425-1439 [PMID: 27375790 DOI: 10.7150/tno.15359]
- Lin M, Chen JF, Lu YT, Zhang Y, Song J, Hou S, Ke Z, Tseng HR. Nanostructure embedded microchips for detection, isolation, and characterization of circulating tumor cells. *Acc Chem Res* 2014; **47**: 2941-2950 [PMID: 25111636 DOI: 10.1021/ar5001617]
- Lu Y, Liang H, Yu T, Xie J, Chen S, Dong H, Sinko PJ, Lian S, Xu J, Wang J, Yu S, Shao J, Yuan B, Wang L, Jia L. Isolation and characterization of living circulating tumor cells in patients by immunomagnetic negative enrichment coupled with flow cytometry. *Cancer* 2015; **121**: 3036-3045 [PMID: 25945459 DOI: 10.1002/cncr.29444]
- Qian W, Zhang Y, Chen W. Capturing Cancer: Emerging Microfluidic Technologies for the Capture and Characterization of Circulating Tumor Cells. *Small* 2015; **11**: 3850-3872 [PMID: 25993898 DOI: 10.1002/smll.201403658]
- Zheng S, Lin HK, Lu B, Williams A, Datar R, Cote RJ, Tai YC. 3D microfilter device for viable circulating tumor cell (CTC) enrichment from blood. *Biomed Microdevices* 2011; **13**: 203-213 [PMID: 20978853 DOI: 10.1007/s10544-010-9485-3]
- Ankeny JS, Court CM, Hou S, Li Q, Song M, Wu D, Chen JF, Lee T, Lin M, Sho S, Rochefort MM, Girgis MD, Yao J, Wainberg ZA, Muthusamy VR, Watson RR, Donahue TR, Hines OJ, Reber HA, Graeber TG, Tseng HR, Tomlinson JS. Circulating tumour cells as a biomarker for diagnosis and staging in pancreatic cancer. *Br J Cancer* 2016; **114**: 1367-1375 [PMID: 27300108 DOI: 10.1038/bjc.2016.121]
- Wang FB, Yang XQ, Yang S, Wang BC, Feng MH, Tu JC. A higher number of circulating tumor cells (CTC) in peripheral blood indicates poor prognosis in prostate cancer patients--a meta-analysis. *Asian Pac J Cancer Prev* 2011; **12**: 2629-2635 [PMID: 22320965]
- Barriere G, Riouallon A, Renaudie J, Tartary M, Rigaud M. Mesenchymal characterization: alternative to simple CTC detection in two clinical trials. *Anticancer Res* 2012; **32**: 3363-3369 [PMID: 22843916]
- Karl A, Tritschler S, Hofmann S, Stief CG, Schindlbeck C. Perioperative search for circulating tumor cells in patients undergoing radical cystectomy for bladder cancer. *Eur J Med Res* 2009; **14**: 487-490 [PMID: 19948444]
- Coumans F, Terstappen L. Detection and Characterization of Circulating Tumor Cells by the CellSearch Approach. *Methods Mol Biol* 2015; **1347**: 263-278 [PMID: 26374323 DOI: 10.1007/978-1-4939-2990-0\_18]
- Huang X, Gao P, Song Y, Sun J, Chen X, Zhao J, Xu H, Wang Z. Meta-analysis of the prognostic value of circulating tumor cells detected with the CellSearch System in colorectal cancer. *BMC Cancer* 2015; **15**: 202 [PMID: 25880692 DOI: 10.1186/s12885-015-1218-9]
- Steinarsdóttir M, Jónasson JG, Vidarsson H, Júlíusdóttir H, Hauksdóttir H, Ögmundsdóttir HM. Cytogenetic changes in nonmalignant breast tissue. *Genes Chromosomes Cancer* 2004; **41**: 47-55 [PMID: 15236316 DOI: 10.1002/gcc.20055]
- Gorges TM, Tinhofer I, Drosch M, Röse L, Zollner TM, Krahn T, von Ahsen O. Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. *BMC Cancer* 2012; **12**: 178 [PMID: 22591372 DOI: 10.1186/1471-2407-12-178]
- Grover PK, Cummins AG, Price TJ, Roberts-Thomson IC, Hardingham JE. Circulating tumour cells: the evolving concept and the inadequacy of their enrichment by EpCAM-based methodology for basic and clinical cancer research. *Ann Oncol* 2014; **25**: 1506-1516 [PMID: 24651410 DOI: 10.1093/annonc/mdl018]
- Gires O, Stoecklein NH. Dynamic EpCAM expression on circulating and disseminating tumor cells: causes and consequences. *Cell Mol Life Sci* 2014; **71**: 4393-4402 [PMID: 25103341 DOI: 10.1007/s00018-014-1693-1]
- Sieuwerds AM, Kraan J, Bolt J, van der Spoel P, Elstrodt F, Schutte M, Martens JW, Gratama JW, Sleijfer S, Foekens JA. Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. *J Natl Cancer Inst* 2009; **101**: 61-66 [PMID: 19116383 DOI: 10.1093/jnci/djn419]
- Diepenbruck M, Christofori G. Epithelial-mesenchymal transition (EMT) and metastasis: yes, no, maybe? *Curr Opin Cell Biol* 2016; **43**: 7-13 [PMID: 27371787 DOI: 10.1016/j.ccb.2016.06.002]
- Limasale YD, Tezcaner A, Özen C, Keskin D, Banerjee S. Epidermal growth factor receptor-targeted immunoliposomes for delivery of celecoxib to cancer cells. *Int J Pharm* 2015; **479**: 364-373 [PMID: 25595386 DOI: 10.1016/j.ijpharm.2015.01.016]
- Baas JM, Krens LL, Guchelaar HJ, Morreau H, Gelderblom H. Concordance of predictive markers for EGFR inhibitors in primary tumors and metastases in colorectal cancer: a review. *Oncologist* 2011; **16**: 1239-1249 [PMID: 21742964 DOI: 10.1634/



- theoncologist.2011-0024]
- 29 **Zhai J**, Scoble JA, Li N, Lovrecz G, Waddington LJ, Tran N, Muir BW, Coia G, Kirby N, Drummond CJ, Mulet X. Epidermal growth factor receptor-targeted lipid nanoparticles retain self-assembled nanostructures and provide high specificity. *Nanoscale* 2015; **7**: 2905-2913 [PMID: 25516406 DOI: 10.1039/c4nr05200e]
  - 30 **Nobuto H**, Sugita T, Kubo T, Shimose S, Yasunaga Y, Murakami T, Ochi M. Evaluation of systemic chemotherapy with magnetic liposomal doxorubicin and a dipole external electromagnet. *Int J Cancer* 2004; **109**: 627-635 [PMID: 14991586 DOI: 10.1002/ijc.20035]
  - 31 **Kullberg M**, Mann K, Owens JL. Improved drug delivery to cancer cells: a method using magnetoliposomes that target epidermal growth factor receptors. *Med Hypotheses* 2005; **64**: 468-470 [PMID: 15617850 DOI: 10.1016/j.mehy.2004.07.033]
  - 32 **Kulshrestha P**, Gogoi M, Bahadur D, Banerjee R. In vitro application of paclitaxel loaded magnetoliposomes for combined chemotherapy and hyperthermia. *Colloids Surf B Biointerfaces* 2012; **96**: 1-7 [PMID: 22521681 DOI: 10.1016/j.colsurfb.2012.02.029]
  - 33 **Onuki Y**, Obata Y, Kawano K, Sano H, Matsumoto R, Hayashi Y, Takayama K. Membrane Microdomain Structures of Liposomes and Their Contribution to the Cellular Uptake Efficiency into HeLa Cells. *Mol Pharm* 2016; **13**: 369-378 [PMID: 26709741 DOI: 10.1021/acs.molpharmaceut.5b00601]
  - 34 **Liang X**, Shi B, Wang K, Fan M, Jiao D, Ao J, Song N, Wang C, Gu J, Li Z. Development of self-assembling peptide nanovesicle with bilayers for enhanced EGFR-targeted drug and gene delivery. *Biomaterials* 2016; **82**: 194-207 [PMID: 26763734 DOI: 10.1016/j.biomaterials.2015.12.015]
  - 35 **Fleischer CC**, Payne CK. Nanoparticle-cell interactions: molecular structure of the protein corona and cellular outcomes. *Acc Chem Res* 2014; **47**: 2651-2659 [PMID: 25014679 DOI: 10.1021/ar500190q]
  - 36 **Moghimi SM**, Hunter AC, Murray JC. Long-circulating and target-specific nanoparticles: theory to practice. *Pharmacol Rev* 2001; **53**: 283-318 [PMID: 11356986]
  - 37 **Driemel C**, Kremling H, Schumacher S, Will D, Wolters J, Lindenlauf N, Mack B, Baldus SA, Hoya V, Pietsch JM, Panagiotidou P, Raba K, Vay C, Vallböhmer D, Harréus U, Knoefel WT, Stoecklein NH, Gires O. Context-dependent adaption of EpCAM expression in early systemic esophageal cancer. *Oncogene* 2014; **33**: 4904-4915 [PMID: 24141784 DOI: 10.1038/onc.2013.441]
  - 38 **Shigdar S**, Qian C, Lv L, Pu C, Li Y, Li L, Marappan M, Lin J, Wang L, Duan W. The use of sensitive chemical antibodies for diagnosis: detection of low levels of EpCAM in breast cancer. *PLoS One* 2013; **8**: e57613 [PMID: 23460885 DOI: 10.1371/journal.pone.0057613]
  - 39 **Yoshida GJ**, Saya H. EpCAM expression in the prostate cancer makes the difference in the response to growth factors. *Biochem Biophys Res Commun* 2014; **443**: 239-245 [PMID: 24309103 DOI: 10.1016/j.bbrc.2013.11.093]
  - 40 **Alves S**, Castro L, Fernandes MS, Francisco R, Castro P, Priault M, Chaves SR, Moyer MP, Oliveira C, Seruca R, Côrte-Real M, Sousa MJ, Preto A. Colorectal cancer-related mutant KRAS alleles function as positive regulators of autophagy. *Oncotarget* 2015; **6**: 30787-30802 [PMID: 26418750 DOI: 10.18632/oncotarget.5021]
  - 41 **Chang YY**, Lin JK, Lin TC, Chen WS, Jeng KJ, Yang SH, Wang HS, Lan YT, Lin CC, Liang WY, Chang SC. Impact of KRAS mutation on outcome of patients with metastatic colorectal cancer. *Hepatology* 2014; **61**: 1946-1953 [PMID: 25713893]
  - 42 **Therkildsen C**, Bergmann TK, Henriksen-Schnack T, Ladelund S, Nilbert M. The predictive value of KRAS, NRAS, BRAF, PIK3CA and PTEN for anti-EGFR treatment in metastatic colorectal cancer: A systematic review and meta-analysis. *Acta Oncol* 2014; **53**: 852-864 [PMID: 24666267]
  - 43 **Wang S**, Hüttmann G, Scholzen T, Zhang Z, Vogel A, Hasan T, Rahmanzadeh R. A light-controlled switch after dual targeting of proliferating tumor cells via the membrane receptor EGFR and the nuclear protein Ki-67. *Sci Rep* 2016; **6**: 27032 [PMID: 27246531 DOI: 10.1038/srep27032]
  - 44 **Mourtzikou A**, Stamouli M, Kroupis C, Christodoulou S, Skondra M, Kastania A, Pectasides D, Athanasas G, Dimas C. Evaluation of carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), epithelial cell adhesion molecule EpCAM (GA733-2), and carbohydrate antigen 19-9 (CA 19-9) levels in colorectal cancer patients and correlation with clinicopathological characteristics. *Clin Lab* 2012; **58**: 441-448 [PMID: 22783573]
  - 45 **Liu J**, Zhou Q, Xu J, Wang J, Zhang Y. Detection of EGFR expression in patients with colorectal cancer and the therapeutic effect of cetuximab. *J BUON* 2016; **21**: 95-100 [PMID: 27061536]
  - 46 **Gorges TM**, Stein A, Quidde J, Hauch S, Röck K, Riethdorf S, Joosse SA, Pantel K. Improved Detection of Circulating Tumor Cells in Metastatic Colorectal Cancer by the Combination of the CellSearch® System and the AdnaTest®. *PLoS One* 2016; **11**: e0155126 [PMID: 27182774 DOI: 10.1371/journal.pone.0155126]
  - 47 **Myung JH**, Launier CA, Eddington DT, Hong S. Enhanced tumor cell isolation by a biomimetic combination of E-selectin and anti-EpCAM: implications for the effective separation of circulating tumor cells (CTCs). *Langmuir* 2010; **26**: 8589-8596 [PMID: 20155985 DOI: 10.1021/la904678p]
  - 48 **Meunier A**, Hernández-Castro JA, Turner K, Li K, Veres T, Juncker D. Combination of Mechanical and Molecular Filtration for Enhanced Enrichment of Circulating Tumor Cells. *Anal Chem* 2016; **88**: 8510-8517 [PMID: 27442305 DOI: 10.1021/acs.analchem.6b01324]
  - 49 **Bhana S**, Wang Y, Huang X. Nanotechnology for enrichment and detection of circulating tumor cells. *Nanomedicine (Lond)* 2015; **10**: 1973-1990 [PMID: 26139129 DOI: 10.2217/nnm.15.32]
  - 50 **Hanssen A**, Wagner J, Gorges TM, Taenzler A, Uzunoglu FG, Driemel C, Stoecklein NH, Knoefel WT, Angenendt S, Hauch S, Atanackovic D, Loges S, Riethdorf S, Pantel K, Wikman H. Characterization of different CTC subpopulations in non-small cell lung cancer. *Sci Rep* 2016; **6**: 28010 [PMID: 27302574 DOI: 10.1038/srep28010]
  - 51 **Tulley S**, Zhao Q, Dong H, Pearl ML, Chen WT. Vita-Assay™ Method of Enrichment and Identification of Circulating Cancer Cells/ Circulating Tumor Cells (CTCs). *Methods Mol Biol* 2016; **1406**: 107-119 [PMID: 26820949 DOI: 10.1007/978-1-4939-3444-7\_9]

**P- Reviewer:** Kane S, McCarthy M, Provenzale D **S- Editor:** Ma YJ  
**L- Editor:** Wang TQ **E- Editor:** Huang Y



Basic Study

# Hypoxia preconditioning protects $\text{Ca}^{2+}$ -ATPase activation of intestinal mucosal cells against R/I injury in a rat liver transplantation model

Zhi-Peng Ji, Yuan-Xin Li, Bao-Xu Shi, Zhuo-Nan Zhuang, Jing-Yan Yang, Sen Guo, Xiao-Zhou Xu, Ke-Sen Xu, Hai-Lin Li

Zhi-Peng Ji, Department of General Surgery, the Second Hospital of Shandong University, Jinan 250033, Shandong Province, China

Yuan-Xin Li, Zhuo-Nan Zhuang, Department of Gastrointestinal Surgery, Beijing Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, Beijing 102218, China

Bao-Xu Shi, Department of Neurology, People's Hospital of Rizhaolanshan, Rizhao 276800, Shandong Province, China

Sen Guo, Department of Hepatobiliary Surgery, Qilu Hospital, Shandong University, Jinan 250033, Shandong Province, China

Xiao-Zhou Xu, Ke-Sen Xu, Hai-Lin Li, Department of Hepatobiliary Surgery, the Second Hospital of Shandong University, Shandong University, Jinan 250033, Shandong Province, China

Jing-Yan Yang, Department of Pathology, the Second Hospital of Shandong University, Jinan 250033, Shandong Province, China

ORCID number: Zhi-Peng Ji (0000-0002-6541-1244); Yuan-Xin Li (0000-0001-8176-822X); Bao-Xu Shi (0000-0001-6148-2662); Zhuo-Nan Zhuang (0000-0002-0881-2631); Jing-Yan Yang (0000-0001-7556-0326); Sen Guo (0000-0003-0175-3963); Xiao-Zhou Xu (0000-0002-0601-4871); Ke-Sen Xu (0000-0003-2168-8214); Hai-Lin Li (0000-0001-5150-3693).

**Author contributions:** Ji ZP and Li HL designed the research and drafted and revised the paper; Ji ZP and Zhuang ZN performed the research; Shi BX, Guo S, Xu XZ and Zhuang ZN searched the literature and analysed the data; Xu KS and Li HL revised the paper and approved the final version; Yang JY provided technical assistance with pathology analysis.

**Supported by** The Second Hospital of Shandong University Youth Foundation, No. Y2013010033.

**Institutional review board statement:** The study was reviewed and approved by The Second Hospital of Shandong University Institutional Review Board.

**Conflict-of-interest statement:** We declare that there are no conflicts of interest to disclose.

**Data sharing statement:** No additional data are available.

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**Manuscript source:** Unsolicited manuscript

**Correspondence to:** Hai-Lin Li, MD, PhD, Adjunct Professor, Department of Hepatobiliary Surgery, the Second Hospital of Shandong University, Shandong University, 247#, Beiyuan Street, Jinan 250033, Shandong Province, China. [lehaln01@163.com](mailto:lehaln01@163.com)

**Telephone:** +86-531-82169203

**Fax:** +86-531-82169243

**Received:** October 17, 2017

**Peer-review started:** October 17, 2017

**First decision:** October 31, 2017

**Revised:** November 7, 2017

**Accepted:** November 27, 2017

**Article in press:** November 27, 2017

**Published online:** January 21, 2018

## Abstract

### AIM

To investigate the effect of ischaemia and reperfusion (I/R) injury on the  $\text{Ca}^{2+}$ -ATPase activation in the intestinal tissue of a rat autologous orthotopic liver

transplantation model and to determine if hypoxia preconditioning (HP) therapy induces HIF-1 $\alpha$  to protect rat intestinal tissue against I/R injury.

## METHODS

Rats received non-lethal hypoxic preconditioning therapy to induce HIF-1 $\alpha$  expression. We used an autologous orthotopic liver transplantation model to imitate the I/R injury in intestinal tissue. Then, we detected the microstructure changes in small intestinal tissues, Ca<sup>2+</sup>-ATPase activity, apoptosis, and inflammation within 48 h postoperatively.

## RESULTS

HIF-1 $\alpha$  expression was significantly increased in intestinal tissue at 12 h postoperatively in rats that were exposed to a hypoxic environment for 90 min compared with a non-HP group (HP *vs* AT,  $P = 0.0177$ ). Pathological analysis was performed on the intestinal mucosa cells, and the cells in the HP group appeared healthier than the cells in the AT group. The Ca<sup>2+</sup>-ATPase activity in the small intestinal cells in the AT group was significantly lower after the operation, and the Ca<sup>2+</sup>-ATPase activity in the HP group recovered faster than that in the AT group at 6 h postoperatively (HP *vs* AT,  $P = 0.0106$ ). BCL-2 expression in the HP group was significantly higher than that in the AT group at 12 h postoperatively (HP *vs* AT  $P = 0.0010$ ). The expression of the inflammatory factors NO, SOD, IL-6, and TNF- $\alpha$  was significantly lower in the HP group than in the AT group.

## CONCLUSION

Hypoxia-induced HIF-1 $\alpha$  could protect intestinal mucosal cells against mitochondrial damage after I/R injury. HP could improve hypoxia tolerance in small intestinal mucosal cells and increase Ca<sup>2+</sup>-ATPase activity to reduce the apoptosis of and pathological damage to intestinal cells. HP could be a useful way to promote the earlier recovery of intestinal function after graft procedure.

**Key words:** Hypoxic precondition; Intestinal function; Ischemia/reperfusion; Liver transplantation; Rat

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**Core tip:** Ischaemia/reperfusion (I/R) injury affects the recovery of postoperative bowel function in liver transplantation. In our research, hypoxia-induced HIF-1 $\alpha$  expression could protect mitochondrial function and Ca<sup>2+</sup>-ATPase activity against I/R injury to reduce the apoptosis and pathological damage to intestinal cells. Therefore, we suggest that hypoxic preconditioning therapy could improve the tolerance of small intestinal mucosal cell to hypoxia in rat autologous orthotopic liver transplantation.

Xu KS, Li HL. Hypoxia preconditioning protects Ca<sup>2+</sup>-ATPase activation of intestinal mucosal cells against R/I injury in a rat liver transplantation model. *World J Gastroenterol* 2018; 24(3): 360-370 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i3/360.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i3.360>

## INTRODUCTION

During liver transplantation, intestinal ischaemia/reperfusion (I/R) injury is usually caused by the blockage of blood flow in the portal vein (PV). The digestive tract usually plays an important role in the pathophysiological process of trauma and shock due to its unique physiological environment, metabolic factors, network of mucosal blood vessels, and counter-current exchange mechanism. In particular, intestinal mucosal I/R injury is associated with systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) after shock or trauma<sup>[1]</sup>. The postoperative recovery of small intestinal mucosa cells is important in treatment and prognosis.

Under normal circumstances, the intestinal mucosa plays an important role in maintaining normal barrier function, absorbing nutrients, and resisting the invasion of local bacteria and toxins in the intestine<sup>[2]</sup>. The intestinal villus nutrient vessels resemble a hairpin that sits at the top of the intestinal villi, and they are extremely curved. Due to their high metabolism and villus microvascular structure characteristics, the tolerance of intestinal mucosa cells to I/R injury, especially the top of the intestinal villus epithelial cells, is much lower than that in other tissue cells; thus, these cells are particularly sensitive to hypo-perfusion<sup>[3]</sup>. Another cause of intestinal mucosal damage is the presence of hypoxia and acidosis in the gastrointestinal tract. Therefore, the intestinal villi can undergo ischaemic damage.

Non-lethal hypoxic preconditioning (HP) can increase tolerance to I/R injury and is effective in reducing damage to a variety of organs<sup>[4]</sup>, including the liver and kidney<sup>[5]</sup>. For tumour cells, research has found that HIF-1 $\alpha$  plays an important role in hypoxia conditioning, and HIF-1 $\alpha$  is also the critical transcription factor that mediates cell hypoxia reactions<sup>[6]</sup>. In our previous study, we induced HIF-1 $\alpha$  expression in liver tissue by exposing rats to a non-lethal hypoxia environment, and detected changes in the NF- $\kappa$ B and Erk pathways. Moreover, changes in glucose metabolism were also detected, and hypoxia-induced HIF-1 $\alpha$  expression promoted HK2 and Glut1 expression, which could decrease liver inflammation and I/R injury after orthotopic liver transplantation. BCL-2 (B-cell lymphoma 2), encoded in humans by the *Bcl-2* gene, is the founding member of the Bcl-2 family of regulator proteins that regulate cell death (apoptosis), by either inducing (pro-apoptotic) or inhibiting (anti-apoptotic) apoptosis<sup>[7,8]</sup>.

Ji ZP, Li YX, Shi BX, Zhuang ZN, Yang JY, Guo S, Xu XZ,

BCL-2 is considered an important anti-apoptotic protein. Studies of human cancer cells also confirmed that the expression of BCL-2 was positively correlated with VEGF and HIF1A, which are target genes of miR-27a and miR-17, and the expression of VEGF and HIF1A was related to the poor prognosis of patients<sup>[9]</sup>. The BCL2 protein functions as an antiapoptotic protein and inhibits programmed cell death<sup>[10]</sup>. Both gene amplification and translocation are common mechanisms for BCL2 protein overexpression in diffuse large B-cell lymphoma (DLBCL). The clinical significance of BCL2 protein expression in DLBCL is still controversial<sup>[11]</sup>.  $\text{Ca}^{2+}$ -ATPase damage is one of the early manifestations in intestinal mucosa cells during ischaemia-reperfusion injury. During intestinal ischaemia, calcium mobilization and extracellular calcium influx greatly increase the calcium ion concentration in the cytoplasm. Intracellular calcium activates proteolytic enzymes, which produce a large number of free radicals that are involved in cell injury during reperfusion. Oxygen free radicals damage cell membrane lipids, and the peroxidation of calcium channels can lead to the inactivation of the  $\text{Na}^{+}$ - $\text{K}^{+}$ -ATPase enzyme and the  $\text{Ca}^{2+}$ / $\text{Na}^{+}$  exchange, which can enhance  $\text{Ca}^{2+}$  influx, leading to intracellular calcium overload<sup>[12]</sup>. Cell damage is caused by mitochondrial oxygen utilization and the synthesis of ATP is further damaged. Acidic products are produced during anaerobic metabolism, which leads to a change in intracellular enzyme activity and a deficient transmembrane ion gradient. When the duration of tissue ischaemia exceeds a certain critical value, I/R injury will be irreversible and the tissue will become necrotic<sup>[13]</sup>.

In this study, we investigated how I/R injury affects the  $\text{Ca}^{2+}$ -ATPase activation in intestinal tissue in a rat autologous orthotopic liver transplantation model. Hypoxia-induced HIF-1 $\alpha$  could protect against the I/R injury to mitochondria and preserve  $\text{Ca}^{2+}$ -ATPase activity in rat intestinal tissue. HP can improve the tolerance of small intestine mucosal cells to hypoxia, and reduce the apoptosis by increasing BCL2 expression and pathological damage to intestinal cells. It could be a useful way to promote the earlier recovery of intestinal function after graft procedure.

## MATERIALS AND METHODS

### Materials

Healthy 8-10-week-old male SD rats that weighed 225-275 g were provided by the Experimental Animal Center of Jiangsu Province. All studies were approved by our Institutional Animal Care and Use Committee. The homemade hypoxic device (referring to Vannucci's and other methods<sup>[4]</sup>) consisted of a noninvasive vascular folder of 8% nitrogen-oxygen mixed gas (containing 8% oxygen and 92% nitrogen, provided by the Nanjing Flextronics Gas Co., Ltd.). Hypoxia equipment consisted of a high pressure tank filled with nitrogen-oxygen mixed gas, a 5-L sealed hypoxic disposal tank,

an outflow of gas, water bottle, mask, oxygen valve, an oxygen flow metre, and connection tubes. The rats can be completely enclosed in the transparent low-disposal tank. Rats were maintained at atmospheric pressure for 90 min with an 8% nitrogen-oxygen gas mixture (containing 8% oxygen and 92% nitrogen) at a flow rate of 5 L/min. Eight hours later, we administered anaesthesia and began the autologous orthotopic liver transplantation surgery.

### Autologous orthotopic liver transplantation

The healthy SD rats were randomly divided into three groups as follows: a normal control group (NC;  $n = 3$ , total  $n = 18$ ), an autotransplantation group (AT;  $n = 3$ , total  $n = 18$ ), or a HP group (HP;  $n = 3$ , total  $n = 18$ ). The autologous orthotopic liver transplantation procedure was performed as follows: after rats were injected with 100 mg/kg ketamine and 0.03 mg atropine intraperitoneally, they were maintained on semi-open mask inhalation of ether for 10 min. After the abdominal cavity was opened, the falciform ligament was resected, and the blood vessel along the oesophagus was removed. The liver was dissected until the suprahepatic vena cava (SVC) was completely liberated. A homemade leash was prepared to guide the SVC for blockage. The PV was dissected from the convergence of the inferior mesenteric and splenic veins. The hepatic artery and biliary tract were freed together. Vascular clamps were used at the convergence of the inferior mesenteric and splenic veins, hepatic artery, SVC, and IVC. The PV was punctured with a No. 4 transfixion pin in preparation for reperfusion, and fixed with a vascular clamp. Ringer's lactate solution was injected for reperfusion at 2.5 mL/min, and a 1-mm incision was made in the wall of the IVC as an outflow tract. The liver gradually turned yellow when reperfusion was successful. A total of 20-25 min passed to imitate the operation range of liver transplantation and the duration of ischaemia/reperfusion injury. At the end of the procedure, the rats received an injection of 1.6 million units of penicillin in the abdomen and 4 mL of Ringer's lactate was infused through the abdominal wall vein, then the abdomen was sutured. After surgery, rats were kept in a 38 °C incubator<sup>[5]</sup>.

### Western blot analysis

Intestinal tissue samples were lysed and cell lysates were collected with radioimmuno-precipitation assay (RIPA) protein lysate buffer. The cytoplasm was centrifuged at 750  $g$  at 4 °C for 5 min, then the supernatant was collected and centrifuged at 13000 rpm at 4 °C for 30 min to collect the sediment. A total of 30  $\mu\text{g}$  of protein were loaded and run on a gel and then transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, United States) for 1 h at 300 mA, followed by blocking with 5% non-fat dry milk in 0.1% TBST for 1 h. The membrane was incubated



with primary antibodies overnight at 4 °C. The primary antibodies against HIF-1 $\alpha$ , Caspase 3, cleaved Caspase 3, and cleaved PARP were purchased from Cell Signalling Technology (United States) and diluted 1:500. The secondary antibodies and  $\beta$ -actin antibody were purchased from Abcam Biotechnology Company.

#### **Histology and immunohistochemistry**

Formalin-fixed paraffin-embedded tissue sections were subjected to immunostaining using the streptavidin-peroxidase technique, with diaminobenzidine as a chromogen. Haematoxylin and eosin (H and E) staining and immunohistochemistry were performed according to standard procedures. The protein expression of BCL-2 was evaluated by immunohistochemistry. The target protein was detected *via* the Elivison two-step immunohistochemical method. Briefly, tissues were dewaxed in xylene and hydrated using an alcohol gradient (ethanol, 95% ethanol in water, 85% ethanol in water, and 70% ethanol in water). A high-temperature plastic staining tray submerged in a beaker of antigen retrieval buffer was used for antigen retrieval. After endogenous peroxidase was inactivated, the primary was added. The sections were then incubated in 50  $\mu$ L of universal IgG antibody-Fab-HRP polymer for 30 min. Subsequently, the glass slides underwent colour development, dehydration, and sealing. The expression of BCL-2 protein was represented by a blue colour in the cells. Image-Pro Plus 6.0 software was used to analyse the optical density of immunohistochemical results. In brief, the scoring was as follows: 0, <10% of cells stained; 1, 10% to 25% of cells stained; 2, 25% to 50% of cells stained; 3, > 50% of cells stained. For all cases, slides with a score  $\geq 2$  were considered positive.

#### **Ca<sup>2+</sup>-ATPase activity analysis**

ATPase (Ca<sup>2+</sup>-ATPase) activity was detected in 0.2 mL of the supernatant added to 0.8 mL of normal saline. One ATPase activity unit per hour is the amount of 1  $\mu$ mol of inorganic phosphorus produced by the decomposition of ATP by per milligram of protein.

#### **Enzyme-linked immunosorbent assay**

Blood samples were collected from rats at 0, 2, 6, 12, 24 and 48 h after the operation and centrifuged to separate sera, which were stored at -80 °C. All serum samples were analysed with ELISA assay kits, which were purchased from Kaiji Biology, Inc. (Nanjing, China). Nitric oxide (NO), superoxide dismutase (SOD), interleukin-6 (IL-6), and rat tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) ELISA kits were purchased from Kaiji Biology Company, Nanjing, China.

#### **Statistical analysis**

Statistical analyses were performed with commercially available SPSS version 19.0 software (Chicago, IL, United States) and GraphPad Prism software (La

Jolla, CA, United States). All experimental data were analysed *via* analysis of variance and are expressed as mean  $\pm$  SD. Independent *t*-tests were used to analyse the differences between groups. *P* < 0.05 was considered statistically significant.

## **RESULTS**

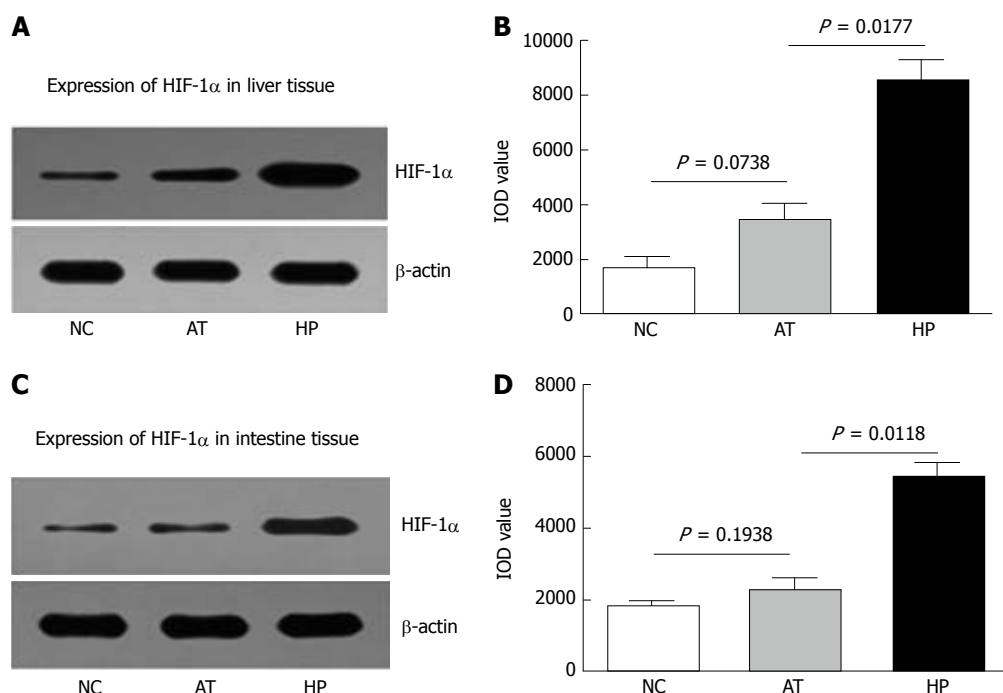
### ***HP induces HIF-1 $\alpha$ expression in liver and intestinal tissues in a rat autologous orthotopic liver transplantation model***

The rats in the HP group were exposed to a hypoxic environment for 90 min before the procedure; this experimental protocol had been used in our lab previously<sup>[14]</sup>. Then, the rats underwent autologous orthotopic liver transplantation. The changes in HIF-1 $\alpha$  levels in the total cellular extract of liver and intestinal tissues were detected 12 h after the procedure. HIF-1 $\alpha$  expression induced by HP was increased in rat liver tissue 12 h after the operation (Figure 1A). We also observed that HIF-1 $\alpha$  expression in the AT group was higher than in the NC group, but the difference was not significant (AT vs NC, *P* = 0.0738, Figure 1B). The increased HIF-1 $\alpha$  expression in ischaemia and hypoxia tissues may have been caused by the procedure, which includes clamping and blocking the blood circulation during the liver transplantation. HIF-1 $\alpha$  expression was significantly higher after the HP therapy than in the non-HP group (HP vs AT, *P* = 0.0177, Figure 1B).

The intestinal tissues were also examined for changes in HIF-1 $\alpha$  levels 12 h after the procedure *via* Western blot analysis. We observed that HIF-1 $\alpha$  expression in intestinal tissues was significantly higher after HP therapy than in the non-HP group (HP vs AT, *P* = 0.0118, Figure 1D). HIF-1 $\alpha$  level in the AT group was not significantly different compared to the NC group (AT vs NC, *P* = 0.1938, Figure 1D).

### ***Changes in intestinal morphology and Ca<sup>2+</sup>-ATPase activity***

Intestinal cells, particularly mucosal cells, usually suffer I/R injury during the liver transplantation procedure. In this study, we collected rat intestinal tissues for pathological examination at 12 h after the operation. The intestinal mucosal cells in the AT group exhibited noticeable oedema, and capillary vessels were filled with red blood cells and blood clots. The villus epithelial cells were shedding, and glands were severely damaged and infiltrated with inflammatory cells (Figure 2B). Mitochondria are important cellular organs that affect the oxidative respiratory chain, and damage to mitochondria that occurs during the early stage of ischaemia/reperfusion injury leads to cell apoptosis. We observed that the mitochondria in the AT group appeared swollen, round, and degenerated 12 h after the operation, and the visible cristae appeared less fractured or even disappeared (Figure 2E). Our previous study showed that HP induced HIF-1 $\alpha$  expression



**Figure 1** Expression of HIF-1 $\alpha$  in liver and intestinal tissues in a rat autologous orthotopic liver transplantation model. A: Western blot assay showed that the level of HIF-1 $\alpha$  protein expression induced by hypoxia preconditioning was increased in rat liver tissue at 12 h postoperatively; B: The chart shows that the level of HIF-1 $\alpha$  in the HP group was higher at different postoperative time points. At 12 h postoperatively, HIF-1 $\alpha$  expression in liver tissue in the HP group was significantly higher than in the AT group and NC group; C: Western blot assay showed that HIF-1 $\alpha$  expression was increased in rat intestinal tissue after hypoxia preconditioning at 12 h postoperatively; D: The chart shows that HIF-1 $\alpha$  expression induced by hypoxia preconditioning in intestinal tissue was elevated at different postoperative time points. HIF-1 $\alpha$  expression was significantly higher in the HP group compared with the AT group and NC group at 12 h postoperatively.

and decreased oxidative respiratory chain damage in mitochondria and protected against I/R injury in liver tissue<sup>[14]</sup>. In this study, we also observed that the intestinal cells appeared to have slight oedema, fewer inflammatory cells were present, and less damage was present in rat intestinal tissue after HP therapy compared with the AT group (Figure 2C). The mitochondria of intestinal cells appeared less swollen with fewer cristae. The structure of the endoplasmic reticulum was maintained (Figure 2F). The intestinal cells and mitochondria appeared normal in the NC group (Figure 2A and D).

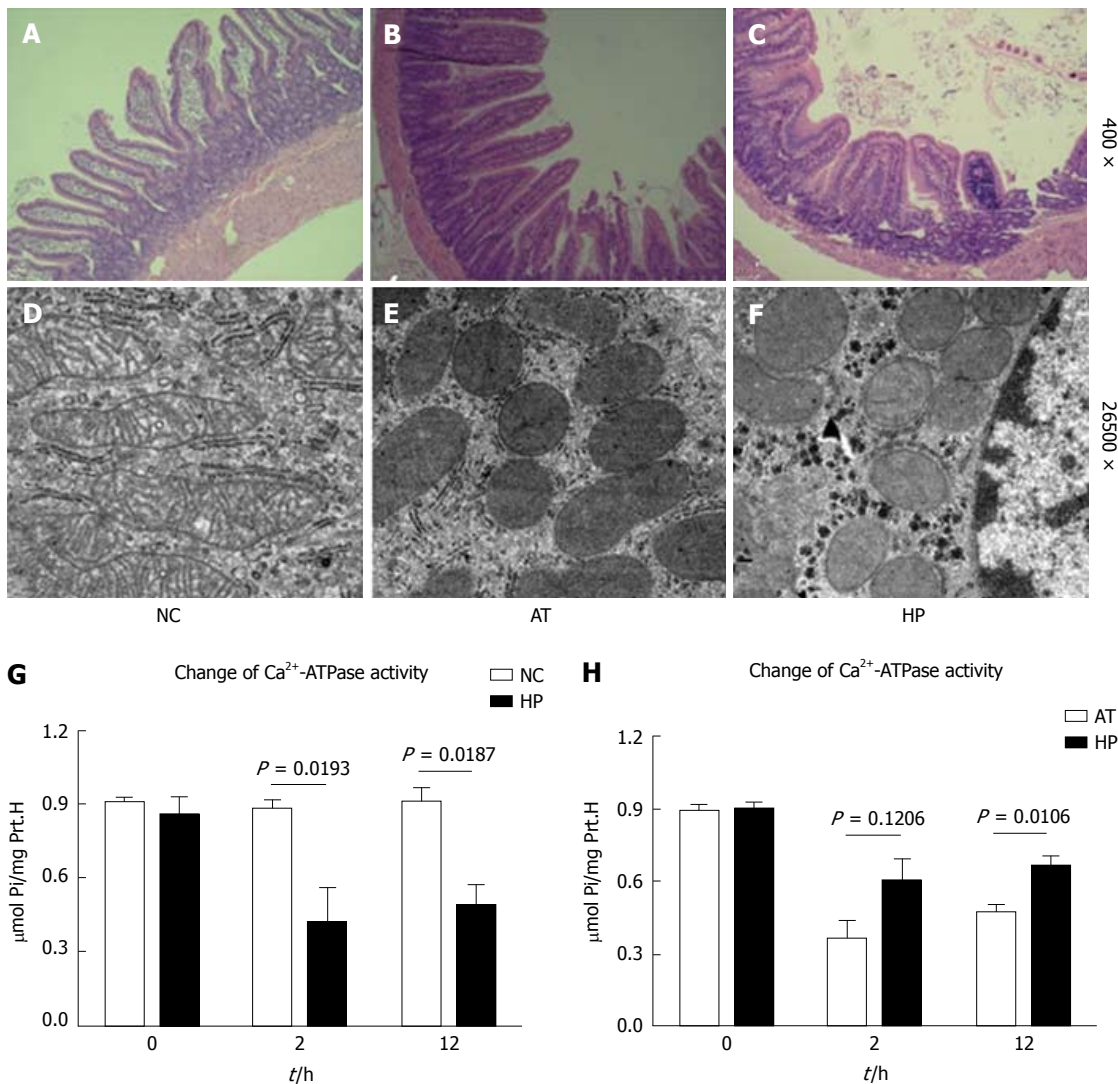
The I/R injury to the intestinal tissue during the procedure may decrease Ca<sup>2+</sup>-ATPase activity, which was evaluated to assess the damage to intestinal cell membranes and to predict functional recovery.

Ca<sup>2+</sup>-ATPase activity in the AT group was significantly lower compared with the NC group, which decreased to the lowest level 2 h postoperatively (Figure 2G, AT vs NC,  $P = 0.0193$ ) and then recovered. The same pattern was evident in the HP group, which exhibited decreased cellular Ca<sup>2+</sup>-ATPase activity after the procedure. However, we observed that the cellular Ca<sup>2+</sup>-ATPase activity in the HP group was significantly higher than that in the AT group 12 h postoperatively (Figure 2H, HP vs AT,  $P = 0.0106$ ). To further detect the effect of HP on the activity of cellular Ca<sup>2+</sup>-ATPase, we analysed the enzyme bioactivity changes at different time points. Table 1 shows that the cellular Ca<sup>2+</sup>-ATPase activity was significantly higher in the

HP group than in the AT group at 2, 12 and 24 h postoperatively (Table 1).

#### **HP promotes BCL2 expression and decreases apoptosis in response to I/R injury in rat intestinal tissues**

Our previous study showed that HP therapy induced HIF-1 $\alpha$  expression and could protect against mitochondrial damage and apoptosis in liver tissue<sup>[14]</sup>. In this study, rat intestinal tissues were analysed by immunohistochemistry to detect BCL2 expression, which can affect cell apoptosis by regulating mitochondrial membrane permeability. Immunohistochemistry demonstrated that the ratio of BCL2 positive signal in the HP group was significantly higher than in the AT group at postoperative 12 h (Figure 3B, HP vs AT  $P = 0.0010$ ). The ratio of positive BCL2 signal was detected at several time points after the operation (2, 6, 12, 24 and 48 h) in the NC, AT and HP groups. The expression of BCL2 in the HP group was increased after the operation, peaked at 12 h postoperatively, and was maintained at a high level. It was significantly higher in the HP group compared with the NC group and the AT group (Figure 3C, 6 h: HP vs AT,  $P = 0.0407$ ; 12 h: HP vs AT,  $P = 0.0301$ ). However, for the AT group, we observed that there was no significant difference in BCL2 expression compared with the NC group (Figure 3C, 6 h: AT vs NC,  $P = 0.0544$ ). To confirm that BCL2 expression inhibited apoptosis in rat intestinal tissue, we detected the cleaved Caspase 3 and cleaved PARP expression levels by Western blot and found that



**Figure 2** Hypoxia-induced HIF-1 $\alpha$  expression increases intestinal cellular mitochondrial integrity and  $\text{Ca}^{2+}$ -ATPase activity after I/R injury. A-C: Pathological changes in intestinal cells are shown (HE, 10  $\times$  20). A: The normal morphology of intestinal cells in the NC group; B: The intestinal cells in the AT group exhibited oedema and red blood cell deposition and blood clots in capillary vessels; the villus epithelial cells were shedding, had severely damaged glands, and had been infiltrated by inflammatory cells; C: The intestinal cells in the HP group exhibited slight oedema and fewer inflammatory cells. The villus epithelial cells were less damaged. D-F: The mitochondrial changes in intestinal cells are shown ( $\times$  46000). D: The normal morphology of mitochondria in the NC group; E: The mitochondria in the AT group appeared swollen, round, and degenerated. The visible mitochondrial cristae appeared less fractured or disappeared; F: The mitochondria in the HP group showed less swelling and fewer cristae. The endoplasmic reticulum structure had survived; G: The  $\text{Ca}^{2+}$ -ATPase activity in the AT group was significantly lower compared with the NC group, which decreased to the lowest level at postoperative 2 h (AT group vs NC group:  $P < 0.05$ ) and then recovered; H: The  $\text{Ca}^{2+}$ -ATPase activity in the HP group was lower after the operation but was significantly higher than that in the AT group at postoperative 2 h (HP group vs AT group:  $P < 0.05$ ).

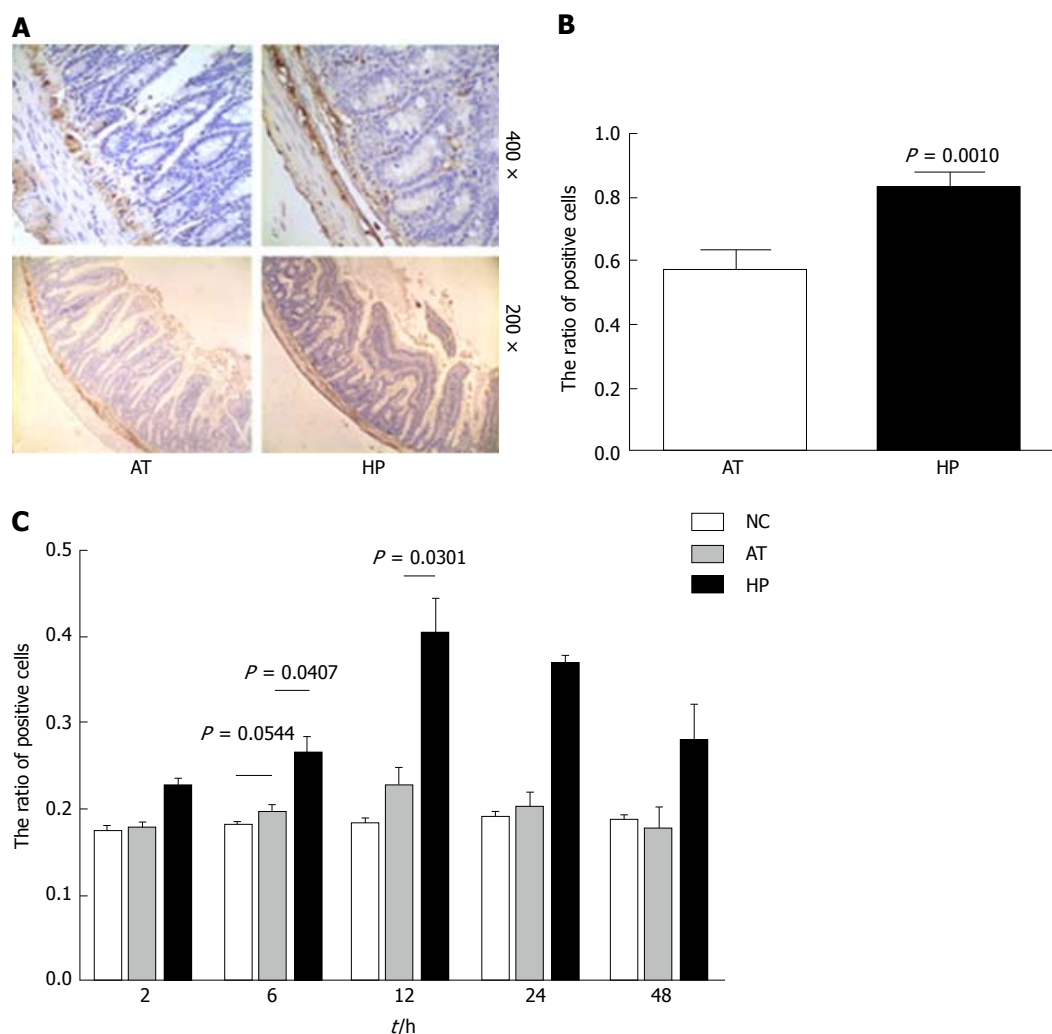
**Table 1**  $\text{Ca}^{2+}$ -ATPase activity in intestinal cells (mean  $\pm$  SD)

Group	n	1 h	2 h	12 h	24 h	48 h
HP	12	0.923 $\pm$ 0.008 <sup>a</sup>	0.389 $\pm$ 0.014 <sup>a,c</sup>	0.566 $\pm$ 0.013 <sup>a,c</sup>	0.771 $\pm$ 0.011 <sup>ac</sup>	0.908 $\pm$ 0.014 <sup>a</sup>
AT	12	0.904 $\pm$ 0.06 <sup>c</sup>	0.323 $\pm$ 0.018 <sup>e</sup>	0.374 $\pm$ 0.011 <sup>e</sup>	0.575 $\pm$ 0.033 <sup>c</sup>	0.871 $\pm$ 0.012 <sup>e</sup>
NC	12	0.923 $\pm$ 0.004	0.916 $\pm$ 0.010	0.928 $\pm$ 0.034	0.932 $\pm$ 0.011	0.909 $\pm$ 0.010
F		3.075	501.688	616.699	274.498	3.109
P value		0.0600	< 0.001	< 0.001	< 0.001	0.0580

<sup>a</sup> $P < 0.05$  vs AT group; <sup>c</sup> $P < 0.05$  vs NC group; <sup>e</sup> $P < 0.05$ , AT group vs NC group.

cleaved Caspase 3 expression was increased in rat intestinal tissue that had undergone I/R injury at 24 h postoperatively. In the AT group, the level of cleaved Caspase 3 was significantly higher than that in the

NC group (Figure 4B, AT vs NC,  $P = 0.0004$ ), while cleaved Caspase 3 expression in the HP group was lower than that in the AT group at postoperative 24 h (Figure 4B, HP vs AT,  $P = 0.0038$ ). The expression of



**Figure 3 Hypoxia preconditioning promotes BCL2 expression in rat intestinal tissues.** A: Immunohistochemistry revealed the expression of BCL2 in rat intestinal tissue in the AT and HP groups at postoperative 12 h; B: The ratio of BCL2 expression in the HP group was significantly higher than that in the AT group at postoperative 12 h (HP vs AT  $P = 0.001$ ); C: The ratio of positive BCL2 expression was detected at several time points postoperatively (2, 6, 12, 24 and 48 h) in the NC, AT, and HP groups. The expression of BCL2 in the HP group was increased after the operation, peaked at postoperative 2 h, and was maintained at a high level. At postoperative 12 h, BCL2 expression in the HP group was significantly higher than that the NC group and the AT group. No significant difference in BCL2 expression was found in the AT group and NC group.

cleaved PARP was increased in rat intestinal tissue that had undergone I/R injury at 24 h postoperatively. The level of cleaved PARP in the AT group was significantly higher than that in the NC group (Figure 4D, AT vs NC,  $P < 0.0001$ ). Cleaved PARP expression in the HP group was lower than that in the AT group at postoperative 24 h (Figure 4D, HP vs AT,  $P = 0.0001$ ).

#### Changes in the levels of serum inflammation factors in the rat autologous orthotopic liver transplantation model

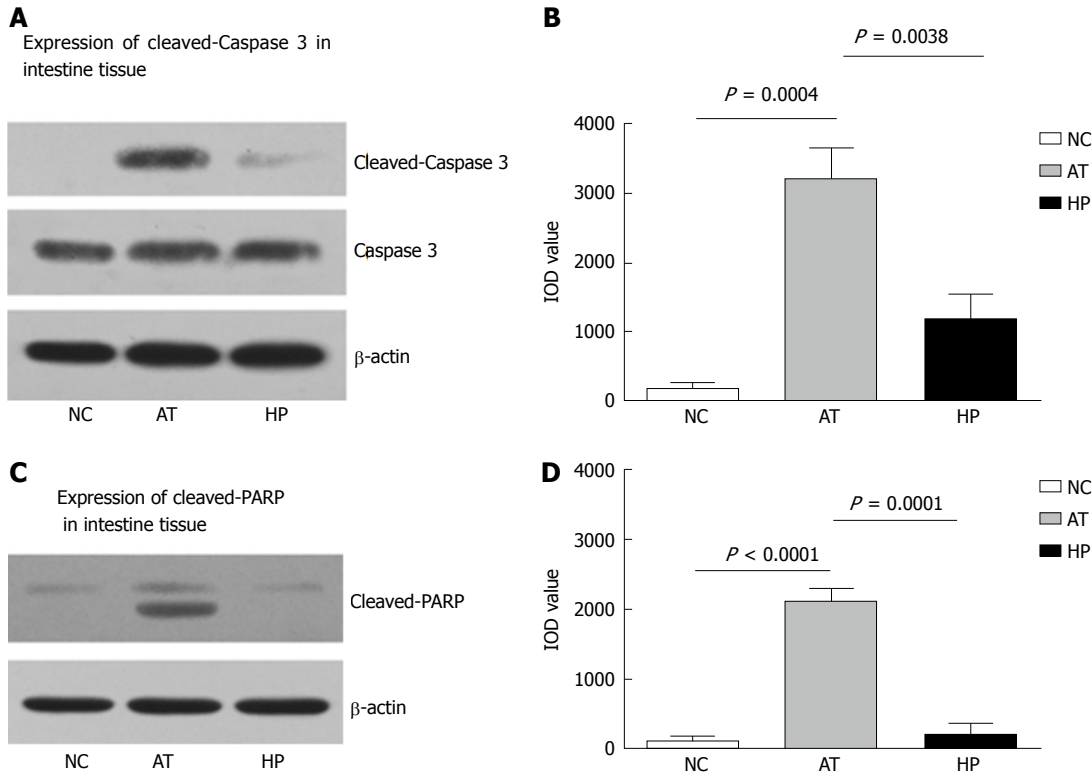
To investigate the ability of HP treatment to protect intestinal tissue from I/R injury, we monitored changes in the levels of NO, SOD, IL-6 and TNF- $\alpha$  in rat serum samples. In the HP group, serum NO and SOD levels increased after the operation, but they were lower compared with the AT group, in which NO and SOD levels stayed elevated after the operation (Figure 5A and B; NO, 24 h: AT vs NC,  $P = 0.0260$ ; AT vs HP,  $P$

$= 0.0083$ ; HP vs NC,  $P = 0.0599$ ; SOD, 24 h: AT vs NC,  $P = 0.0357$ ; HP vs AT,  $P = 0.0352$ ; HP vs NC,  $P = 0.2036$ ). We measured the serum levels of IL-6 and TNF- $\alpha$ , and found that HP treatment appeared to relieve some of the inflammatory reaction due to the lower IL-6 and TNF- $\alpha$  levels that were detected (Figure 5C and D, IL-6, 24 h: AT vs NC,  $P < 0.0001$ ; AT vs HP,  $P = 0.0097$ ; HP vs NC,  $P = 0.0113$ ; TNF- $\alpha$ , 24 h: AT vs NC,  $P < 0.0001$ ; HP vs AT,  $P = 0.0029$ ; HP vs NC,  $P = 0.0007$ ).

## DISCUSSION

There is a close relationship between the contractile activity of the small intestine and the intracellular concentration of  $\text{Ca}^{2+}$ [15].  $\text{Ca}^{2+}$ -ATPase can be found in the plasma membrane, endoplasmic reticulum, and mitochondrial membrane. It can be activated and hydrolyse ATP to provide energy when the





**Figure 4** Changes of Caspase 3 and PARP expression in rat intestinal tissue at postoperative 24 h. A: The apoptosis was detected by Western blot assay, which showed that the expression of cleaved Caspase 3 as increased in rat intestinal tissue with ischemia and reperfusion injury at postoperative 24 h; B: The bar-table shows the level of cleaved Caspase 3 expression in AT group was significantly higher than that in the NC group. The cleaved Caspase 3 expression in the HP group was lower than that in the AT group at postoperative 24 h; C: The expression of cleaved PARP was increased in rat intestinal tissue with ischemia and reperfusion injury at postoperative 24 h; D: The bar-table shows that the level of cleaved PARP expression in the AT group was significantly higher than that in the NC group. The cleaved PARP expression in the HP group was lower than that in the AT group at postoperative 24 h.

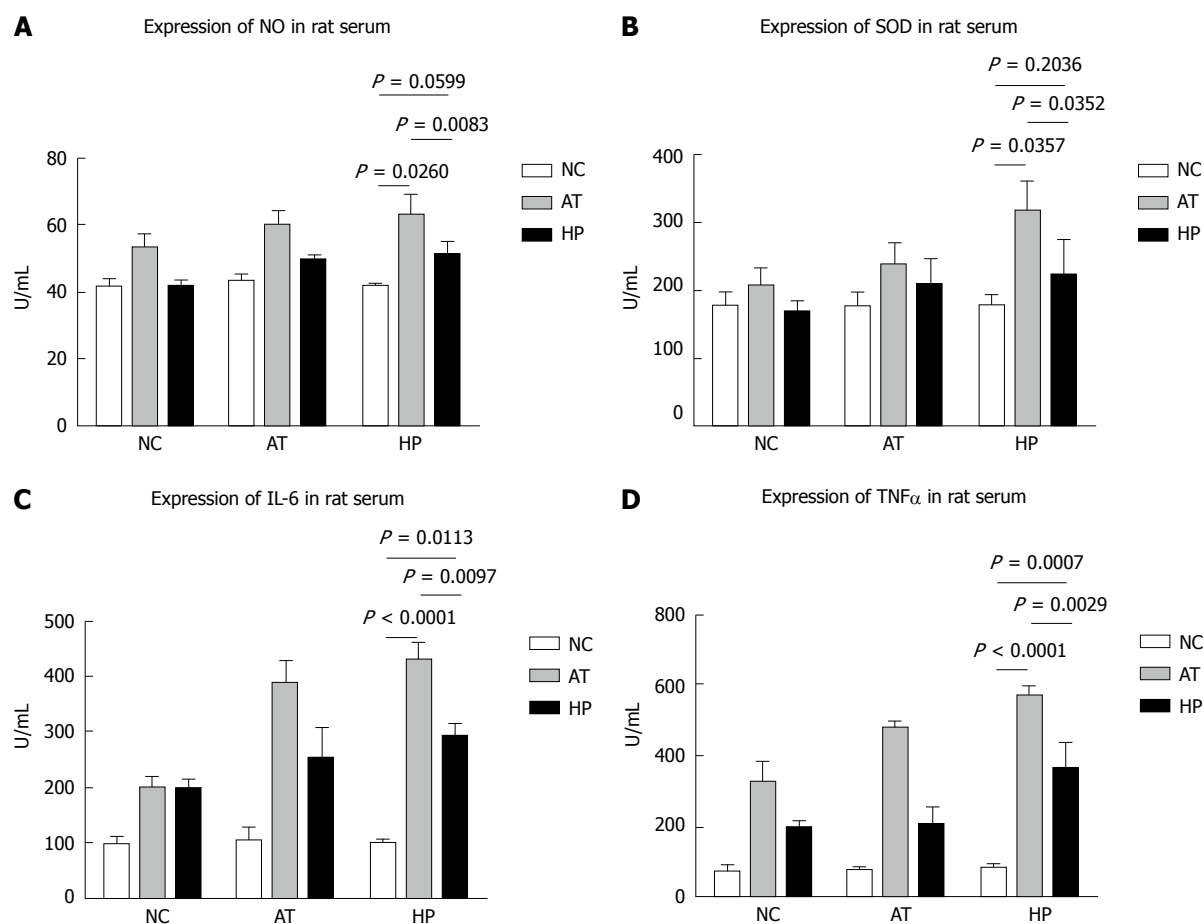
concentration of  $\text{Ca}^{2+}$  rises to a certain level. Therefore,  $\text{Ca}^{2+}$ -ATPase levels can indicate ischaemia-reperfusion injury because they can reflect the severity of cell injury<sup>[16]</sup>. The results of the present study showed that the intestine suffered hypoxia with congestion after the occlusion of the PV during autologous orthotopic liver transplantation. The calcium balance system became dysfunctional because the activity of the  $\text{Ca}^{2+}$ -ATPase in intestinal tissue was damaged due to the presence of toxins in the blood. We found that the  $\text{Ca}^{2+}$ -ATPase activity in intestinal tissue which suffered the I/R injury after the procedure of autologous orthotopic liver transplantation was significantly lower compared with the non-surgery intestinal tissue. Therefore, the systolic and diastolic function of the intestinal smooth muscle was inhibited in the autologous orthotopic liver transplantation group, resulting in decreased peristalsis of the stomach and intestine.

Due to hypoxia, intestinal epithelial cells were shedding and necrosis occurred, increasing intestinal permeability and triggering intestinal bacteria and endotoxin translocation<sup>[17]</sup>. In a previous study, we showed that HIF-1 $\alpha$  promoted HK2 and Glut1 expression could decrease the liver inflammation and I/R injury after orthotopic liver transplantation<sup>[14]</sup>. In this study, due to non-noticeably reduced  $\text{Ca}^{2+}$ -ATPase activity in the liver transplantation group with HPtherapy, small intestinal

tissue exhibited less apoptosis and hypoxia tolerance was increased after I/R injury and exposure to oxygen free radicals and toxins in the intestine. Our previous study found that HP protects mitochondria against I/R injury<sup>[18]</sup>. In this study, we observed that  $\text{Ca}^{2+}$ -ATPase activity in intestinal tissue of the rat with HP therapy was not noticeably reduced compared to the non-surgery intestinal tissue. The cells could adapt to hypoxia and post-operative injury in a hypoxic environment was reduced.

BCL2 affects cell apoptosis by regulating mitochondrial membrane permeability. The mechanism may be that BCL2 changes the pores or channels in the mitochondrial membrane<sup>[19-21]</sup>. BCL2 is considered an important anti-apoptotic protein, and it can affect cell apoptosis by regulating mitochondrial membrane permeability.

Our previous studies found that HP was sufficient to activate the BCL2 signalling pathway and up-regulated the expression of BCL2 protein, a regulatory factor that restrains apoptosis, and it may regulate apoptosis by altering the configuration of mitochondria in liver tissue of the rat liver transplantation model<sup>[22]</sup>. In this study, HP therapy protected the rat intestinal tissue against apoptosis as revealed by a low level of cleaved Caspase 3 and cleaved PARP expression after the liver transplantation procedure compared to the



**Figure 5 Hypoxia preconditioning decreases the expression of inflammatory factors in rat serum after postoperative I/R injury of intestinal tissue.** A: In the HP group, NO levels in the serum were lower, especially at 12 h and 24 h after the operation, compared with the AT group; B: The SOD levels in the HP group 12 h and 24 h after the operation were significantly lower than those in the AT group; C: After the operation, the IL-6 levels in rat liver tissues that had undergone ischaemia-reperfusion injury were significantly higher in the AT group compared with the HP group and the NC group; D: TNF- $\alpha$  levels in the HP group were slightly decreased compared with those in the AT group.

rat with non-HP therapy. BCL2 expression was also increased in the HP therapy group and may be related with Ca<sup>2+</sup>-ATPase activity and mitochondrial protection after intestinal I/R injury in the rat autologous liver transplantation model. It requires further studies to show the relationship between HP and BCL2.

During HP, intestinal mucosal cells could be resistant to hypoxia caused by the blockage of blood flow during liver transplant, which would reduce apoptosis. HP therapy protected the intestinal mucosa cells against I/R injury. The characteristic morphological changes of intestinal mucosa cells undergoing HP therapy included slight oedema and fewer inflammatory cells. In addition, the tops of the epithelial cells were less damaged, the mitochondria showed less swelling and fewer cristae, and the endoplasmic reticulum structure was visible. However, the situation was worse in the autologous orthotopic liver transplantation group; cells were filled with red blood cells and blood clots in capillary vessels, intestinal mucosa cells exhibited obvious oedema, and infiltrated inflammatory cells surrounded the epithelial cells. The villus epithelial cells were shedding, had severely damaged glands, and

had been infiltrated by inflammatory cells. The swollen mitochondria appeared round, had degenerated, and had severe visible cristae that had fewer fractures or had disappeared.

In summary, the early recovery of enteral nutrition is very important for intestinal prognosis after liver transplantation. It is essential to reduce the damage to small intestinal mucosal cells. In this study, we used a rat autologous orthotopic liver transplantation model to simulate intestinal I/R injury and observed that HP could protect Ca<sup>2+</sup>-ATPase and reduce small intestinal mucosal mitochondrial damage and apoptosis. The protective mechanism of Ca<sup>2+</sup>-ATPase against intestinal cristae I/R injury in rat autologous liver transplantation requires further study. I/R injury was decreased in the intestine, and inflammation was reduced. These findings may provide a theoretical basis for the clinical recovery and treatment in liver transplantation, but further experimental evidence is needed.

## ARTICLE HIGHLIGHTS

### Research background

During liver transplantation, intestinal ischaemia/reperfusion (I/R) injury

usually occurs due to the blockage of blood flow in the portal vein. Intestinal mucosal I/R injury is related with SIRS and MODS after shock or trauma. The postoperative recovery of small intestinal mucosal cells is important in treatment and prognosis. Non-lethal hypoxic preconditioning (HP) can increase tolerance to I/R injury and is effective in reducing damage to a variety of organs. In our previous study, we induced HIF-1 $\alpha$  expression in liver tissue by exposing rats to a non-lethal hypoxia environment, and detected changes in the NF- $\kappa$ B and Erk pathways. Moreover, changes in glucose metabolism were also detected, and hypoxia-induced HIF-1 $\alpha$  expression promoted HK2 and Glut1 expression, which could decrease liver inflammation and I/R injury after orthotopic liver transplantation. BCL-2 is considered an important anti-apoptotic protein. Ca<sup>2+</sup>-ATPase damage is one of the early manifestations in intestinal mucosa cells during ischaemia-reperfusion injury.

### Research motivation

In this study, we investigated how I/R injury affects the Ca<sup>2+</sup>-ATPase activation in intestinal tissue in a rat autologous orthotopic liver transplantation model.

### Research objectives

To investigate the effect of I/R injury on the Ca<sup>2+</sup>-ATPase activation in rat intestinal tissue in a rat autologous orthotopic liver transplantation model and to determine if HP therapy induced HIF-1 $\alpha$  to protect rat intestinal tissue against I/R injury.

### Research methods

Non-lethal hypoxic preconditioning therapy was applied to induce HIF-1 $\alpha$  expression. An autologous orthotopic liver transplantation model was established to imitate I/R injury to intestinal tissue. Then, we detected the microstructure changes in small intestinal tissues using histology and immunohistochemistry, the expression of HIF-1 $\alpha$ , cleaved Caspase 3, and cleaved PARP by Western blot analysis, and the expression of inflammatory factors in rat serum by ELISA.

### Research results

After HP therapy, HIF-1 $\alpha$  expression was significantly increased in intestinal tissue of rats at 12 h postoperatively. Pathology of the intestinal mucosal cells appeared healthier in the HP group than in the AT group. The Ca<sup>2+</sup>-ATPase activity in small intestinal cells in the HP group recovered faster than that in the AT group. BCL-2 expression in the HP group was significantly higher than that in the AT group. The expression of the inflammatory factors NO, SOD, IL-6 and TNF- $\alpha$  was significantly lower in the HP group than in the AT group.

### Research conclusions

Hypoxia-induced HIF-1 $\alpha$  could protect against the I/R injury to mitochondria and preserve Ca<sup>2+</sup>-ATPase activity in rat intestinal tissue. HP can improve the tolerance of small intestinal mucosal cells to hypoxia, and reduce the apoptosis by increasing BCL2 expression and pathological damage to intestinal cells.

### Research perspectives

Non-lethal HP could be a useful way to promote the earlier recovery of intestinal function after graft procedure.

## REFERENCES

- 1 Fink MP, Delude RL. Epithelial barrier dysfunction: a unifying theme to explain the pathogenesis of multiple organ dysfunction at the cellular level. *Crit Care Clin* 2005; **21**: 177-196 [PMID: 15781156 DOI: 10.1016/j.ccc.2005.01.005]
- 2 Moore-Olufemi SD, Kozar RA, Moore FA, Sato N, Hassoun HT, Cox CS Jr, Kone BC. Ischemic preconditioning protects against gut dysfunction and mucosal injury after ischemia/reperfusion injury. *Shock* 2005; **23**: 258-263 [PMID: 15718925]
- 3 Fujise T, Iwakiri R, Wu B, Amemori S, Kakimoto T, Yokoyama F, Sakata Y, Tsunada S, Fujimoto K. Apoptotic pathway in the rat small intestinal mucosa is different between fasting and ischemia-reperfusion. *Am J Physiol Gastrointest Liver Physiol* 2006; **291**: G110-G116 [PMID: 16574989 DOI: 10.1152/ajpgi.00393.2005]
- 4 Schurr A, Reid KH, Tseng MT, Edmonds HL Jr, West CA, Rigor BM. Effect of electrical stimulation on the viability of the hippocampal slice preparation. *Brain Res Bull* 1986; **16**: 299-301 [PMID: 3697795]
- 5 Liang SH, Tao LD, Zhang PJ, Wang ML, Feng M, Liu XY, Zhang M. The effect of hypoxic preconditioning to rat liver function and organizational structure. *Jiangsu Daxue Xuebao* (Medical Sciences), 2007; **17**: 220-221 [DOI: 10.13312/j.issn.1671-7783.2007.03.010]
- 6 Vannucci RC, Towfighi J, Vannucci SJ. Hypoxic preconditioning and hypoxic-ischemic brain damage in the immature rat: pathologic and metabolic correlates. *J Neurochem* 1998; **71**: 1215-1220 [PMID: 9721747 DOI: 10.1046/j.1471-4159.1998.71031215.x]
- 7 Zou C, Xu Q, Mao F, Li D, Bian C, Liu LZ, Jiang Y, Chen X, Qi Y, Zhang X, Wang X, Sun Q, Kung HF, Lin MC, Dress A, Wardle F, Jiang BH, Lai L. MiR-145 inhibits tumor angiogenesis and growth by N-RAS and VEGF. *Cell Cycle* 2012; **11**: 2137-2145 [PMID: 22592534 DOI: 10.4161/cc.20598]
- 8 Fasanaro P, D'Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G, Capogrossi MC, Martelli F. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. *J Biol Chem* 2008; **283**: 15878-15883 [PMID: 18417479 DOI: 10.1074/jbc.M800731200]
- 9 Maniotis AJ, Folberg R, Hess A, Seftor EA, Gardner LM, Pe'er J, Trent JM, Meltzer PS, Hendrix MJ. Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am J Pathol* 1999; **155**: 739-752 [PMID: 10487832 DOI: 10.1016/S0002-9440(10)65173-5]
- 10 Nichols AC, Finkelstein DM, Faquin WC, Westra WH, Mroz EA, Kneuerz P, Begum S, Michaud WA, Busse PM, Clark JR, Rocco JW. Bcl2 and human papilloma virus 16 as predictors of outcome following concurrent chemoradiation for advanced oropharyngeal cancer. *Clin Cancer Res* 2010; **16**: 2138-2146 [PMID: 20233885 DOI: 10.1158/1078-0432.CCR-09-3185]
- 11 Iqbal J, Neppalli VT, Wright G, Dave BJ, Horsman DE, Rosenwald A, Lynch J, Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Campo E, Ott G, Müller-Hermelink HK, Delabie J, Jaffe ES, Grogan TM, Connors JM, Vose JM, Armitage JO, Staudt LM, Chan WC. BCL2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma. *J Clin Oncol* 2006; **24**: 961-968 [PMID: 16418494 DOI: 10.1200/JCO.2005.03.4264]
- 12 Lounsbury KM, Hu Q, Ziegelstein RC. Calcium signaling and oxidant stress in the vasculature. *Free Radic Biol Med* 2000; **28**: 1362-1369 [PMID: 10924855]
- 13 Grace PA. Ischaemia-reperfusion injury. *Br J Surg* 1994; **81**: 637-647 [PMID: 8044536 DOI: 10.1002/bjs.1800810504]
- 14 Zhuonan Z, Sen G, Zhipeng J, Maoyou Z, Linglan Y, Gangping W, Cheng J, Zhongliang M, Tian J, Peijian Z, Kesen X. Hypoxia preconditioning induced HIF-1 $\alpha$  promotes glucose metabolism and protects mitochondria in liver I/R injury. *Clin Res Hepatol Gastroenterol* 2015; **39**: 610-619 [PMID: 25726501 DOI: 10.1016/j.clinre.2014.12.012]
- 15 Schreiber R, Faria D, Skryabin BV, Wanitchakool P, Rock JR, Kunzelmann K. Anoctamins support calcium-dependent chloride secretion by facilitating calcium signaling in adult mouse intestine. *Pflugers Arch* 2015; **467**: 1203-1213 [PMID: 24974903 DOI: 10.1007/s00424-014-1559-2]
- 16 Wei L, Chen WY, Hu T, Tang YX, Pan BB, Jin M, Kong GY. Effect and mechanism of propofol in hepatic ischemia/reperfusion injury of rat. *Eur Rev Med Pharmacol Sci* 2017; **21**: 3516-3522 [PMID: 28829487]
- 17 van der Heijden KM, van der Heijden IM, Galvao FH, Lopes CG, Costa SF, Abdala E, D'Albuquerque LA, Levin AS. Intestinal translocation of clinical isolates of vancomycin-resistant *Enterococcus faecalis* and ESBP-producing *Escherichia coli* in a rat model of bacterial colonization and liver ischemia/reperfusion injury. *PLoS One* 2014; **9**: e108453 [PMID: 25255079 DOI: 10.1371/journal.pone.0108453]

- 18 **Zhuang Z**, Lian P, Wu X, Shi B, Zhuang M, Zhou R, Zhao R, Zhao Z, Guo S, Ji Z, Xu K. Abate Cytochrome C induced apoptosome to protect donor liver against ischemia reperfusion injury on rat liver transplantation model. *Am J Transl Res* 2016; **8**: 1738-1747 [PMID: 27186297]
- 19 **Tsujimoto Y**. Cell death regulation by the Bcl-2 protein family in the mitochondria. *J Cell Physiol* 2003; **195**: 158-167 [PMID: 12652643 DOI: 10.1002/jcp.10254]
- 20 **Kuwana T**, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneider R, Green DR, Newmeyer DD. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* 2002; **111**: 331-342 [PMID: 12419244]
- 21 **Saito M**, Korsmeyer SJ, Schlesinger PH. BAX-dependent transport of cytochrome c reconstituted in pure liposomes. *Nat Cell Biol* 2000; **2**: 553-555 [PMID: 10934477 DOI: 10.1038/35019596]
- 22 **Jin C**, Zhang PJ, Wu XM, Zhou B, Li Y, Liu XY, Feng M, Tao LD. Impact of hypoxic preconditioning on apoptosis and its possible mechanism in orthotopic liver autotransplantation in rats. *Hepatobiliary Pancreat Dis Int* 2009; **8**: 40-45 [PMID: 19208513]

**P-Reviewer:** Berger BM, Cetinkunar S, Gad EH **S-Editor:** Gong ZM  
**L-Editor:** Wang TQ **E-Editor:** Li D





## Case Control Study

# Multi-parameter gene expression profiling of peripheral blood for early detection of hepatocellular carcinoma

Hui Xie, Yao-Qin Xue, Peng Liu, Peng-Jun Zhang, Sheng-Tao Tian, Zhao Yang, Zhi Guo, Hua-Ming Wang

Hui Xie, Sheng-Tao Tian, Zhao Yang, Hua-Ming Wang, Department of Interventional Therapy, 302 Hospital of People's Liberation Army, Beijing 100039, China

Yao-Qin Xue, Zhi Guo, Department of Interventional Therapy, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin, Tianjin's Clinical Research Center for Cancer, Tianjin 300070, China

Yao-Qin Xue, Department of Interventional Therapy, Shanxi Province Cancer Hospital, Shanxi Medical University, Taiyuan 030000, Shanxi Province, China

Peng Liu, Peng-Jun Zhang, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Interventional Therapy Department, Peking University Cancer Hospital and Institute, Beijing 100142, China

**Author contributions:** Xie H, Xue YQ, Liu P, Guo Z and Wang HM designed the study; Xie H, Xue YQ and Liu P performed the research; Xie H, Zhang PJ, Tian ST and Zhao Y analyzed the data; Xie H, Xue YQ and Liu P wrote the paper; Guo Z and Wang HM revised the manuscript for final submission; Xie H, Xue YQ and Liu P contributed equally to this study; Guo Z and Wang HM are the co-corresponding authors.

**Supported by** National Key R&D Program of China, No. 2016YFC0106604; and National Natural Science Foundation of China, No. 81471761 and No. 81501568.

**Institutional review board statement:** The study was reviewed and approved by the 302 Hospital of People's Liberation Army Institutional Review Board.

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

**Conflict-of-interest statement:** We declare that we have no financial or personal relationships with other individuals or organizations that can inappropriately influence our work and that there is no professional or other personal interest of any nature in any product, service and/or company that could be construed

as influencing the position presented in or the review of the manuscript.

**Data sharing statement:** The study participants provided informed consent for data sharing. No additional data are available.

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**Manuscript source:** Unsolicited manuscript

**Correspondence to:** Hua-Ming Wang, BCPS, MD, Chief Doctor, Professor, Department of Interventional Therapy, 302 Hospital of People's Liberation Army, 100 Middle West 4<sup>th</sup> Ring Road, Fengtai District, Beijing 100039, China. [hmwang302@126.com](mailto:hmwang302@126.com)  
Telephone: +86-10-66933263  
Fax: +86-10-66933263

**Received:** August 9, 2017

**Peer-review started:** August 9, 2017

**First decision:** August 29, 2017

**Revised:** October 16, 2017

**Accepted:** November 21, 2017

**Article in press:** November 21, 2017

**Published online:** January 21, 2018

## Abstract

### AIM

In our previous study, we have built a nine-gene (*GPC3*, *HGF*, *ANXA1*, *FOS*, *SPAG9*, *HSPA1B*, *CXCR4*, *PFN1*, and *CALR*) expression detection system based on the GeXP system. Based on peripheral blood and GeXP, we

aimed to analyze the results of genes expression by different multi-parameter analysis methods and build a diagnostic model to classify hepatocellular carcinoma (HCC) patients and healthy people.

## METHODS

Logistic regression analysis, discriminant analysis, classification tree analysis, and artificial neural network were used for the multi-parameter gene expression analysis method. One hundred and three patients with early HCC and 54 age-matched healthy normal controls were used to build a diagnostic model. Fifty-two patients with early HCC and 34 healthy people were used for validation. The area under the curve, sensitivity, and specificity were used as diagnostic indicators.

## RESULTS

Artificial neural network of the total nine genes had the best diagnostic value, and the AUC, sensitivity, and specificity were 0.943, 98%, and 85%, respectively. At last, 52 HCC patients and 34 healthy normal controls were used for validation. The sensitivity and specificity were 96% and 86%, respectively.

## CONCLUSION

Multi-parameter analysis methods may increase the diagnostic value compared to single factor analysis and they may be a trend of the clinical diagnosis in the future.

**Key words:** Hepatocellular carcinoma; Peripheral blood; Early detection; Multi-parameter; Diagnostic value

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**Core tip:** We aimed to analyze the results of expression of nine genes, which we identified previously, by different multi-parameter analysis methods and build a diagnostic model to classify hepatocellular carcinoma patients and healthy people. Logistic regression analysis, discriminant analysis, classification tree analysis, and artificial neural network were used for the multi-parameter gene expression analysis.

Xie H, Xue YQ, Liu P, Zhang PJ, Tian ST, Yang Z, Guo Z, Wang HM. Multi-parameter gene expression profiling of peripheral blood for early detection of hepatocellular carcinoma. *World J Gastroenterol* 2018; 24(3): 371-378 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i3/371.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i3.371>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world<sup>[1]</sup>. Chronic infection with hepatitis B or C virus, dietary aflatoxin B1 intake, and

alcohol abuse clearly show a significant correlation with the incidence of HCC. In China, more than 90% of HCC patients are reported to experience chronic HBV infection<sup>[2]</sup>. Currently, HCC is often diagnosed at an advanced stage and has a poor prognosis. Clinical practice has demonstrated that early diagnosis of HCC can significantly increase the survival time. Many biomarkers have been proposed, and some are currently used in clinical diagnosis<sup>[3,4]</sup>; however, even alpha-fetoprotein, the most widely used biomarker for HCC diagnosis, has a poor diagnostic value<sup>[5]</sup>. Although pathology is used as a gold standard for diagnosis of HCC, it is invasive, and tissue samples are not easily obtained. Therefore, a non-invasive, accurate, and fast method for early detection of HCC is urgently needed.

Peripheral blood samples, which are easily and repeatedly obtained in the clinical setting, have been demonstrated to be valuable for disease prediction and classification, drug response evaluation, and toxicity classification<sup>[6-8]</sup>. These features make peripheral blood samples attractive to aid in the early detection of HCC<sup>[9]</sup>. As we know, HCC is a complex multi-gene and multi-factorial disease, and a single biomarker is not adequate to reflect the HCC status. A panel of biomarkers is a promising method for early detection of HCC, and now some panels have been used for cancer prediction<sup>[10,11]</sup>. A single gene analysis method is not sufficient when gene expression is used for diagnosis. Multi-parameter analysis methods, such as logistic regression analysis (LRA), discriminant analysis (DA), classification tree analysis (CTA), and artificial neural network (ANN), which can analyze multiple factors, have been shown to increase the diagnostic sensitivity and specificity and may be promising analysis methods for multi-parameter analysis<sup>[12-14]</sup>.

In our previous study, we used Affymetrix to screen differential gene expression and built a 9-gene (*GPC3*, *HGF*, *ANXA1*, *FOS*, *SPAG9*, *HSPA1B*, *CXCR4*, *PFN1*, and *CALR*) expression detection system based on the GenomeLab GeXP Genetic Analysis system<sup>[15]</sup>, known as GeXP, which can detect up to 35 genes in one reaction<sup>[16]</sup>. Based on peripheral blood and GeXP, we compared multi-parameter gene expression using various multi-parameter analysis methods and built a diagnostic model to classify early-stage HCC patients and healthy people.

## MATERIALS AND METHODS

### Patients and blood collection

The study was reviewed and approved by the 302 Hospital of People's Liberation Army Institutional Review Board. After obtaining patient consent, blood samples from 103 early-stage HCC patients with chronic HBV infection were collected at our hospital. Fifty-four age-matched healthy normal control samples were collected from the people who underwent a health examination. Both samples were used to build the diagnostic

**Table 1** Characteristics of early-stage hepatocellular carcinoma and healthy control samples

Variable	Building model		Model validation	
	HCC	Control	HCC	Control
Number of patients	103	54	52	34
Male	54	29	29	19
Female	49	25	23	15
Age (mean $\pm$ SD)	54 $\pm$ 12	49 $\pm$ 11	51 $\pm$ 14	52 $\pm$ 9
Tumor size (> 3 cm)	29	0	13	0
Tumor size (< 3 cm)	74	0	39	0
Number of nodules (Unilocular)	36	0	21	0
Number of nodules (Multilocular)	67	0	31	0
Cirrhosis	89	0	36	0
BCLC stage (T1)	27	0	19	0
BCLC stage (T2)	76	0	32	0

HCC: Hepatocellular carcinoma.

model. Fifty-two early-stage HCC patients and 34 healthy people were used for validation. The disease status of early-stage HCC patients was confirmed by histopathological analysis, and tumors were staged according to the Barcelona Clinic Liver Cancer (BCLC) staging classification as either T1 (single lesion < 2 cm in diameter) or T2 (single lesion between 2 and 5 cm in diameter or < 3 lesions, each of which was < 3 cm in diameter)<sup>[17]</sup>. In addition, peripheral blood from HCC patients was collected before any therapy. The clinical characteristics of all the samples used for this study are shown in Table 1.

Peripheral blood (2.5 mL) was collected and added into the PAXGene blood RNA tubes (Qiagen, Valencia, CA, United States). After inverting, the tubes were stored at -80 °C until total RNA extraction.

#### RNA isolation and GeXP gene expression detection

Total RNA was isolated using the PAXGene Blood RNA Kit (Qiagen, Valencia, CA, United States) according to the manufacturer's instructions. The quality and quantity of RNA were measured by agarose gel electrophoresis and DU 800 spectrophotometry (Beckman Coulter, Fullerton, CA, United States), respectively. The primers for the nine genes were shown in our previous study. Reverse and forward primers were diluted 1:200 and 1:500 in nuclease-free water to a final concentration of 500 nmol/L and 200 nmol/L, respectively. Total RNA (50 ng) was used for reverse transcription (RT) with chimeric reverse primers in a single reaction. The concentrations of the primers were diluted at a ratio of 1:8. The RT reactions were performed using the following parameters: 48 °C for 1 min, 42 °C for 60 min, 95 °C for 5 min, and then held at 4 °C.

Polymerase chain reaction (PCR) reaction (10  $\mu$ L) was performed in the 96-well PCR Detection Plate using the following parameters: 95 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 minute. The PCR products were diluted using nuclease-free water and added to the detection plate, which contained sample loading solution and

DNA Size Standard 400. The GeXP system was used to match each gene fragment size and to measure the fluorescent dye signal strength in arbitrary units, and then the dataset was normalized to the housekeeping gene B2M. Finally, the dataset was log transformed.

#### Multi-parameter gene expression analysis

LRA<sup>[18,19]</sup>, DA<sup>[20,21]</sup>, CTA<sup>[22,23]</sup>, and ANN analysis<sup>[24,25]</sup> were used as the multi-parameter gene expression analysis methods. In LRA, the "Forward: Conditional" method was used to select variables. The stepwise probability was "Entry 0.05" and "Removal 0.10", the "Probabilities" was saved, and then "CI for exp (B) 95%" was shown. The "Probabilities" of the HCC group were used for receiver operating characteristic (ROC) analysis, and the cutoff value was based on the Youden Index. In DA, "Use stepwise method" was used to select variables. The stepwise criterion was the "F value". The "Entry" was 3.84, and the "Removal" was 2.71. The "Probabilities of group membership" was saved as a new variable, "Dis\_n," which represented the probability of HCC, and then the "Probabilities of group membership" was used for ROC analysis. In the CTA, "Exhaustive CHAID" was used as a growth method, and 20% of the samples were selected as the validation samples. The "Predicted value" was saved as a new variable for the ROC analysis. In the ANN analysis, the ROC curve was saved, and 80% of the samples were used as the training group and 20% as the test group. After comparison, the best diagnostic model was chosen, and 52 HCC samples and 34 healthy normal controls were used to validate the model. The ROC curve, area under the curve (AUC), sensitivity, and specificity were used as diagnostic indicators.

## RESULTS

#### Genes used for diagnostic evaluation

A total of nine genes (*GPC3*, *HGF*, *ANXA1*, *FOS*, *SPAG9*, *HSPA1B*, *CXCR4*, *PFN1*, and *CALR*) were used for HCC detection. We used the ROC curve of the

**Table 2** Diagnostic value of the four genes showing *P*-values less than 0.05

Gene	AUC	<i>P</i> value	95%CI for AUC		Cutoff	Sen	Spe
			Lower	Upper			
<i>HGF</i>	0.620	0.014	0.532	0.708	0.102	0.408	0.926
<i>ANXA1</i>	0.697	< 0.001	0.614	0.780	0.919	0.728	0.593
<i>SPAG9</i>	0.761	< 0.001	0.681	0.842	0.477	0.942	0.481
<i>PFN1</i>	0.700	< 0.001	0.620	0.781	0.383	0.437	0.907

*P* < 0.05 means significant difference. AUC: Area under curve; Sen: Sensitivity; Spe: Specificity.

**Table 3** Odds ratio of the five selected genes after logistic regression analysis

Gene	<i>P</i>	OR	95% CI for OR	
			Lower	Upper
<i>ANXA1</i>	< 0.001	1.73E+09	1.67E+05	1.79E+13
<i>FOS</i>	0.012	4.80E-03	7.30E-05	3.16E-01
<i>SPAG9</i>	< 0.001	1.36E+14	3.19E+07	5.80E+20
<i>CXCR4</i>	< 0.001	6.44E-02	1.47E-02	2.81E-01
<i>PFN1</i>	< 0.001	1.37E+04	9.82E+01	1.91E+06

*P* < 0.05 means significant difference.

nine genes to evaluate the diagnostic value and then analyzed the *P*-value and 95% confidence interval (CI). The diagnostic value of the AUC, the *P*-value, 95% confidence interval, cutoff value, sensitivity, and specificity are shown in Table 2. The ROC curves from four genes (*HGF*, *ANXA1*, *SPAG9*, and *PFN1*) showed *P*-values less than 0.05. According to the *P*-values, four genes (*HGF*, *ANXA1*, *SPAG9*, and *PFN1*) were chosen to have diagnostic value. In the multi-parameter analysis, both the 9-gene and the 4-gene sets were used.

### Multi-parameter LRA

In the LRA for HCC detection, we compared the diagnostic value of the full 9-gene set (*GPC3*, *HGF*, *ANXA1*, *FOS*, *SPAG9*, *HSPA1B*, *CXCR4*, *PFN1*, and *CALR*) and the 4-gene set (*HGF*, *ANXA1*, *SPAG9*, and *PFN1*). From the full 9-gene set, five genes were selected using a "Forward: Conditional" method. The diagnostic formula was as follows:

$$Y = -4.089 + 21.269 X_{ANXA1} - 5.339 X_{FOS} + 32.543 X_{SPAG9} - 2.743 X_{CXCR4} + 9.524 X_{PFN1} \quad (Y = \text{logit } P)$$

In the 4-gene set, three genes were selected, and the diagnostic formula was as follows:

$$Y = -4.826 + 13.172 X_{ANXA1} + 15.353 X_{SPAG9} + 8.755 X_{PFN1} \quad (Y = \text{logit } P)$$

Then, the probability was used for evaluating the diagnostic value. The ROC curves are shown in Figure 1. The AUC of the five selected genes was 0.933, and that of the three selected genes was 0.878. This indicated that the five selected genes had better diagnostic value, and when the cutoff of logit *P* was 0.548, the sensitivity and specificity were 94% and 80%, respectively. According to the diagnostic formula, we can know the risk of the detected sample. In addition, the odds ratio (OR) of the five selected genes

was also analyzed and is shown in Table 3. An OR greater than 1 indicates that the factor is a risk factor for the disease, and an OR less than 1 means it is a protective factor. Thus, the *ANXA1*, *SPAG9*, and *PFN1* genes were risk factors. When their gene expression is higher, it may increase the incidence of HCC. The *FOS* and *CXCR4* genes were found to be protective factors. When their gene expression is higher, it may decrease the incidence of HCC.

### Multi-parameter DA

In the DA, the full 9-gene set was analyzed by Bayes DA. Six genes were selected by "Use stepwise method". The following formulas were used separately:

$$Y_1 = -7.306 + 8.078 X_{GPC3} + 20.770 X_{ANXA1} - 3.414 X_{FOS} + 12.652 X_{SPAG9} + 1.842 X_{CXCR4} + 18.248 X_{PFN1}$$

$$Y_2 = -5.612 + 15.760 X_{GPC3} + 4.432 X_{ANXA1} + 1.585 X_{FOS} - 10.298 X_{SPAG9} + 4.320 X_{CXCR4} + 12.370 X_{PFN1}$$

In the 4-gene set, three genes entered the diagnostic formulas. The formulas were as follows:

$$Y_1 = -6.665 + 21.814 X_{ANXA1} + 18.524 X_{SPAG9} + 16.663 X_{PFN1}$$

$$Y_2 = -2.806 + 13.784 X_{ANXA1} + 8.081 X_{SPAG9} + 10.393 X_{PFN1}$$

The probability of the healthy normal group and the HCC group analyzed by the three selected genes was saved as a new variable named Dis 1. The probability of the six selected genes was saved as Dis 2. The new variables Dis 1 and Dis 2 were used for the ROC analysis, as shown in Figure 2. The AUC of Dis 1 was 0.877 and of Dis 2 was 0.926. This result meant that the six selected genes had better diagnostic value for HCC, and when the cutoff of *Y* was 0.628, the sensitivity and specificity were 82% and 89%, respectively.

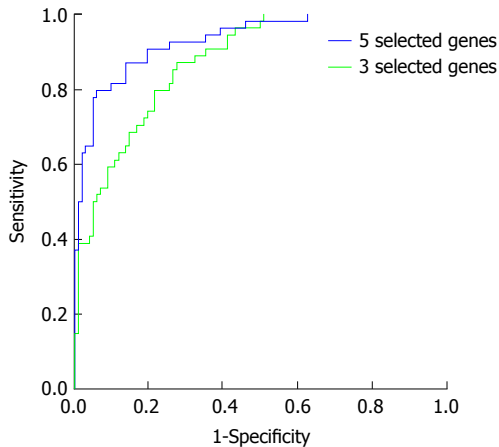
### Multi-parameter CTA

We compared the diagnostic value of the full 9-gene set and the 4-gene set by CTA; however, only *ANXA1* entered the classification tree. The classification tree of the full 9-gene set was the same as the 4-gene set, as shown in Figure 3. When the cutoff was 0.629, the sensitivity and specificity were 70% and 60%, respectively. The total accuracy was only 66.20%.

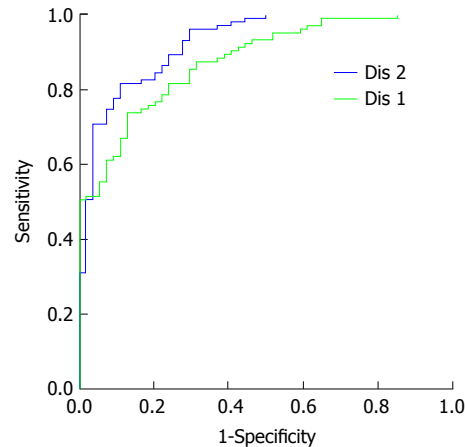
### Multi-parameter ANN analysis

The full 9-gene set and the 4-gene set were then analyzed by the ANN method; their network had one hidden layer which contained five units. In the training





**Figure 1 Receiver operating characteristic analysis of probability after logistic regression analysis.** In the total nine genes, five (*ANXA1*, *FOS*, *SPAG9*, *CXCR4*, and *PFN1*) entered the diagnostic formula. In the four genes, three (*ANXA1*, *SPAG9*, and *PFN1*) entered the formula. The probability was used for ROC analysis. The AUC of the five selected genes was 0.933 and that of the three selected genes was 0.878, indicating that the five selected genes had better diagnostic value, and the sensitivity and specificity were 94% and 80%, respectively.



**Figure 2 Receiver operating characteristic analysis of probability after discriminant analysis.** In the total nine genes, *GPC3*, *ANXA1*, *FOS*, *SPAG9*, *CXCR4*, and *PFN1* genes entered the diagnosis formula. In the four genes, *ANXA1*, *SPAG9*, and *PFN1* genes entered the formula. The probability was used for HCC detection. The AUC of the six selected genes was 0.926 and that of the three selected genes was 0.877. When the cutoff of the five selected genes was 0.628, the sensitivity and specificity were 82% and 89%, respectively.

group of the full 9-gene set, the percent incorrect prediction was 12.3%, and for the testing group, it was 9.1%. In the 4-gene set, the percentages were 20.9% and 8.7%, respectively. In addition, the full 9-gene set had better predictive probabilities than the 4-gene set. The AUC of the full 9-gene set was 0.943, greater than that of the 4-gene set, which was 0.877.

#### Comparison of multiple multi-parameter analysis methods and validation

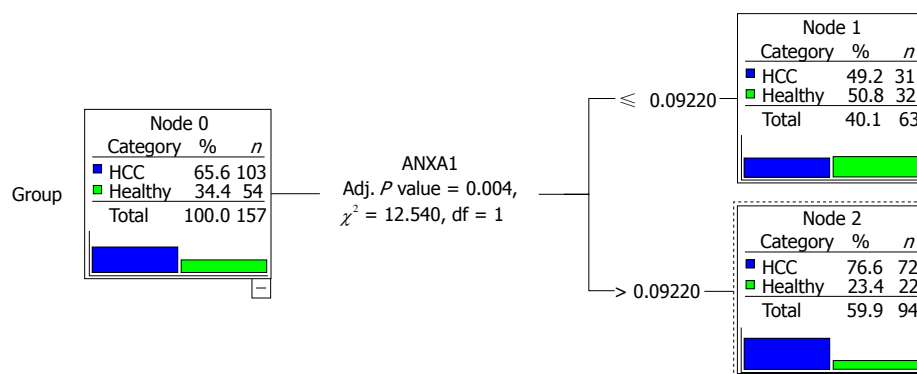
Multiple multi-parameter analysis methods were used to build models, and the AUC was used to evaluate the diagnostic value. When a single gene was used, the AUC of *SPAG9* was greater than those of the other genes. The AUCs of LRA, DA, and ANN were greater than that of *SPAG9*, and the AUC of CTA was less than that of *SPAG9*. Among the methods, the ANN of the full 9-gene set had the best diagnostic value; the AUC, sensitivity, and specificity were 0.943, 98%, and 85%, respectively. Finally, 52 HCC patients and 34 healthy normal controls were used for validation. The sensitivity and specificity were 96% and 86%, respectively. Above all, multi-parameter analysis methods may increase the diagnostic value compared to single-factor analysis and this approach may be a trend for future clinical diagnostic methods.

## DISCUSSION

Clinical peripheral blood samples can be obtained easily and in a minimally invasive way. Studies have shown that mRNA in peripheral blood has the potential to be used for the early detection of cancers. There are many mRNA detection methods; however, the most commonly used method, real-time PCR, is limited by

the number of genes and the amplification efficiency. The Beckman Coulter (Fullerton, CA, United States) GenomeLab GeXP Genetic Analysis system was designed ideally for up to 35 genes per reaction and can be used to detect 192 samples simultaneously in one single detection<sup>[26]</sup>. In addition, the GeXP system uses a universal priming strategy to decrease the variations in amplification efficiency across multiple genes<sup>[16,27]</sup>. Both strengths make GeXP an ideal multiple-gene expression detection method as well as a useful validation tool that is more similar to large-scale gene analysis methods, such as microarrays, than real-time PCR. We combined gene-chip analysis, peripheral blood, the GeXP detection system, and bioinformatics by using gene screening, model building, and bioinformatics analysis to build a gene expression profiling standard operating procedure for the early detection of cancer.

Studies have demonstrated that multi-parameter analysis can increase the sensitivity and specificity and is considered promising for future diagnostic methods. Many multi-parameter methods have been used for cancer early detection. In our study, logistic analysis increased the AUC to 0.933. Out of the nine genes, five (*ANXA1*, *FOS*, *SPAG9*, *CXCR4*, and *PFN1*) entered the diagnostic formula; however, *FOS* and *CXCR4*, which had poor diagnostic value, also entered the formula. This demonstrated that the genes showed significant differences between groups, and even if they had AUC values of less than 0.5, they may contribute to the logistic analysis. In the 4-gene set, three genes (*ANXA1*, *SPAG9*, and *PFN1*) entered the formula, but the AUC was less than that of the full 9-gene set. This demonstrated that genes with more significant differences may result in better diagnostic values.



**Figure 3** Classification tree of the total nine genes and the four genes. The tree of the total nine genes was the same to that of the four genes. Only ANXA1 gene entered the tree, and according to the node, it was divided into two parts.

The logistic had a formula which can get a continuous value: logit (*P*). With a gene expression panel, we can predict the *P*-value to differentiate healthy people and HCC patients, which may provide a clinical indication for both physicians and patients.

After OR analysis, the *ANXA1*, *SPAG9*, and *PFN1* genes were detected as risk factors. The *FOS* and *CXCR4* genes were protective factors. Annexin1 (*ANXA1*) is a member of the annexin family of phospholipid-binding and calcium-binding proteins with a well demonstrated role in early delayed inhibitory feedback of glucocorticoids in the hypothalamus and pituitary gland<sup>[28]</sup>. Studies have demonstrated that *ANXA1* is involved in tumorigenesis and can increase the incidence of HCC<sup>[29]</sup>. *SPAG9* (sperm associated antigen 9) is a gene encoding c-Jun-amino-terminal kinase-interacting protein 4. This enzyme is a scaffolding protein that connects the mitogen-activated protein kinases to related transcription factor targets for the activation of JNK signaling pathways<sup>[30]</sup>. *SPAG9* was also demonstrated to be a biomarker for breast cancer and cervical carcinoma<sup>[31,32]</sup>. Profilin-1 (*PFN1*) has been regarded as a tumor-suppressor molecule for breast cancer, and it can enhance ADP-to-ATP exchange on G-actin. In addition, it can also act as a shuttle to deliver ATP-bound G-actin to facilitate actin polymerization<sup>[33]</sup>. Studies have demonstrated that *PFN1* is overexpressed in cancer cells by up-regulating *PTEN* and down-regulating *AKT*, and it is also an inhibitor of mammary carcinoma aggressiveness<sup>[34]</sup>. If *PFN1* is silenced, it can inhibit endothelial cell proliferation, migration, and morphogenesis<sup>[35]</sup>. All three genes may contribute to the development of HCC.

In the DA, the AUC was similar to the logistic analysis. This demonstrated that it may be a valuable analysis method; however, because it had strict demand on the data distribution, its application was greatly limited. In our study, although we got the discriminant formula, it may have high bias because the dataset was not normally distributed. In the CTA, only one gene had diagnostic value. This finding

demonstrated that they were not suitable for our study. ANN analysis has been demonstrated to provide better diagnostic value in disease prediction and cancer early detection. In our study, ANN had the best diagnostic value compared to the other analysis methods, and the predicted probabilities of the groups were also shown. In addition, in the training group for the full 9-gene set, the percent incorrect prediction was 12.3%, and for the testing group, it was 9.1%. All of these demonstrate that the ANN model we built was successful.

In our previous study, we screened the mRNA in peripheral blood samples by Affymetrix GeneChip analysis, and nine genes (*GPC3*, *HGF*, *ANXA1*, *FOS*, *SPAG9*, *HSPA1B*, *CXCR4*, *PFN1*, and *CALR*) were used for differentiating the healthy normal control and HCC groups. We have now built an ANN detection system. The sensitivity and specificity were 96% and 86%, respectively, which were greater than those of single gene analysis.

## ARTICLE HIGHLIGHTS

### Research background

We have built a 9-gene (*GPC3*, *HGF*, *ANXA1*, *FOS*, *SPAG9*, *HSPA1B*, *CXCR4*, *PFN1* and *CALR*) expression detection system based on the GeXP system in our previous study. We aimed to analyze the results of gene expression by different multi-parameter analysis methods and build a diagnostic model to classify hepatocellular carcinoma (HCC) patients and healthy people.

### Research motivation

Although pathology is used as a golden standard for diagnosis of HCC, it is invasive and tissue sample is not easily obtained. Therefore, a non-invasive, accurate, and fast method for early detection of HCC is pressing.

### Research objectives

A non-invasive, accurate, and fast method for early detection of HCC may be provided by our research based on the mRNA in peripheral blood.

### Research methods

We have successfully built an artificial neural network detection system combining detection system and bioinformatics together for differentiating the healthy normal group and HCC group. The sensitivity and specificity were separately 96% and 86%, respectively, which were greater than those of single-

gene analysis.

### Research results

Artificial neural network of the total nine genes had the best diagnostic value, and the AUC, sensitivity, and specificity were 0.943, 98%, and 85%, respectively. At last, 52 HCC patients and 34 healthy normal controls were used for validation. The sensitivity and specificity were 96% and 86%, respectively.

### Research conclusions

Based on the mRNA in peripheral blood, a multi-parameter analysis method was used to analyze multiple genes, which may increase the diagnostic value compared to the single factor analysis for the early detection of HCC, and it may be a trend of the clinical diagnosis in the future. It may provide a non-invasive, accurate, and fast method for early detection of HCC.

### Research perspectives

The GeXP system uses a universal priming strategy to decrease the variations in amplification efficiency across multiple genes, and it is an ideal multiple-gene expression detection method as well as a useful validation tool that is more similar to large-scale gene analysis methods. Combination of the peripheral blood, GeXP detection system, and bioinformatics together may be the future strategy to build an assistant detection method for cancer.

## REFERENCES

- 1 **Parkin DM**, Bray FI, Devesa SS. Cancer burden in the year 2000. The global picture. *Eur J Cancer* 2001; **37** Suppl 8: S4-S66 [PMID: 11602373]
- 2 **Gao JD**, Shao YF, Xu Y, Ming LH, Wu ZY, Liu GT, Wang XH, Gao WH, Sun YT, Feng XL, Liang LM, Zhang YH, Sun ZT. Tight association of hepatocellular carcinoma with HBV infection in North China. *Hepatobiliary Pancreat Dis Int* 2005; **4**: 46-49 [PMID: 15730918]
- 3 **Berretta M**, Cavaliere C, Alessandrini L, Stanzione B, Facchini G, Balestreri L, Perin T, Canzonieri V. Serum and tissue markers in hepatocellular carcinoma and cholangiocarcinoma: clinical and prognostic implications. *Oncotarget* 2017; **8**: 14192-14220 [PMID: 28077782 DOI: 10.18632/oncotarget.13929]
- 4 **Xu J**, Li J, Zheng TH, Bai L, Liu ZJ. MicroRNAs in the Occurrence and Development of Primary Hepatocellular Carcinoma. *Adv Clin Exp Med* 2016; **25**: 971-975 [PMID: 28028963 DOI: 10.17219/acem/36460]
- 5 **El-Serag HB**, Marrero JA, Rudolph L, Reddy KR. Diagnosis and treatment of hepatocellular carcinoma. *Gastroenterology* 2008; **134**: 1752-1763 [PMID: 18471552 DOI: 10.1053/j.gastro.2008.02.090]
- 6 **Wang Y**, Barbacioru CC, Shiffman D, Balasubramanian S, Iakoubova O, Tranquilli M, Albornoz G, Blake J, Mehmet NN, Ngadimo D, Poulter K, Chan F, Samaha RR, Eleftheriades JA. Gene expression signature in peripheral blood detects thoracic aortic aneurysm. *PLoS One* 2007; **2**: e1050 [PMID: 17940614 DOI: 10.1371/journal.pone.0001050]
- 7 **Kuzman MR**, Medved V, Terzic J, Krainc D. Genome-wide expression analysis of peripheral blood identifies candidate biomarkers for schizophrenia. *J Psychiatr Res* 2009; **43**: 1073-1077 [PMID: 19358997 DOI: 10.1016/j.jpsychires.2009.03.005]
- 8 **Alonso V**, Neves AF, Marangoni K, Faria PC, Freschi AP, Capaneli AC, Meola J, Goulart LR. Gene expression profile in the peripheral blood of patients with prostate cancer and benign prostatic hyperplasia. *Cancer Detect Prev* 2009; **32**: 336-337 [PMID: 19026495 DOI: 10.1016/j.cdp.2008.10.001]
- 9 **Yin CQ**, Yuan CH, Qu Z, Guan Q, Chen H, Wang FB. Liquid Biopsy of Hepatocellular Carcinoma: Circulating Tumor-Derived Biomarkers. *Dis Markers* 2016; **2016**: 1427849 [PMID: 27403030 DOI: 10.1155/2016/1427849]
- 10 **Paczynski S**, Krijanovski OI, Braun TM, Choi SW, Clouthier SG, Kuick R, Misek DE, Cooke KR, Kitko CL, Weyand A, Bickley D, Jones D, Whitfield J, Reddy P, Levine JE, Hanash SM, Ferrara JL. A biomarker panel for acute graft-versus-host disease. *Blood* 2009; **113**: 273-278 [PMID: 18832652 DOI: 10.1182/blood-2008-07-167098]
- 11 **Aarøe J**, Lindahl T, Dumeaux V, Sæbø S, Tobin D, Hagen N, Skaane P, Lönneborg A, Sharma P, Børresen-Dale AL. Gene expression profiling of peripheral blood cells for early detection of breast cancer. *Breast Cancer Res* 2010; **12**: R7 [PMID: 20078854 DOI: 10.1186/bcr2472]
- 12 **Han M**, Liew CT, Zhang HW, Chao S, Zheng R, Yip KT, Song ZY, Li HM, Geng XP, Zhu LX, Lin JJ, Marshall KW, Liew CC. Novel blood-based, five-gene biomarker set for the detection of colorectal cancer. *Clin Cancer Res* 2008; **14**: 455-460 [PMID: 18203981]
- 13 **Liew CC**, Ma J, Tang HC, Zheng R, Dempsey AA. The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool. *J Lab Clin Med* 2006; **147**: 126-132 [PMID: 16503242 DOI: 10.1016/j.lab.2005.10.005]
- 14 **Osman I**, Bajorin DF, Sun TT, Zhong H, Douglas D, Scattergood J, Zheng R, Han M, Marshall KW, Liew CC. Novel blood biomarkers of human urinary bladder cancer. *Clin Cancer Res* 2006; **12**: 3374-3380 [PMID: 16740760 DOI: 10.1158/1078-0432.CCR-05-2081]
- 15 **Zhang PJ**, Run WW, P L, Wang CB, Deng XX, Wang BB, Chen BB, J J, Liu HY, Dong ZN, Zhang XJ, Tian YP. Peripheral blood mRNA expression patterns to differentiate hepatocellular carcinoma from other hepatic diseases. *Front Biosci (Elite Ed)* 2012; **4**: 620-630 [PMID: 22201899 DOI: 10.2741/e404]
- 16 **Rai AJ**, Kamath RM, Gerald W, Fleisher M. Analytical validation of the GeXP analyzer and design of a workflow for cancer-biomarker discovery using multiplexed gene-expression profiling. *Anal Bioanal Chem* 2009; **393**: 1505-1511 [PMID: 18958454 DOI: 10.1007/s00216-008-2436-7]
- 17 **Llovet JM**, Fuster J, Bruix J; Barcelona-Clinic Liver Cancer Group. The Barcelona approach: diagnosis, staging, and treatment of hepatocellular carcinoma. *Liver Transpl* 2004; **10**: S115-S120 [PMID: 14762851 DOI: 10.1002/lt.20034]
- 18 **Zhou X**, Liu KY, Wong ST. Cancer classification and prediction using logistic regression with Bayesian gene selection. *J Biomed Inform* 2004; **37**: 249-259 [PMID: 15465478 DOI: 10.1016/j.jbi.2004.07.009]
- 19 **Elemeery MN**, Badr AN, Mohamed MA, Ghareeb DA. Validation of a serum microRNA panel as biomarkers for early diagnosis of hepatocellular carcinoma post-hepatitis C infection in Egyptian patients. *World J Gastroenterol* 2017; **23**: 3864-3875 [PMID: 28638226 DOI: 10.3748/wjg.v23.i21.3864]
- 20 **Sergeev AS**, Agapova RK, Bogadel'nikova IV, Perel'man MI. [The use of discrete characters in discriminant analysis for diagnosis of pulmonary tuberculosis and for classification of patients differing in treatment efficiency based on polymorphisms at nine codominant loci-HP, GC, TF, PI, PGM1, GLO1, C3, ACP1 and ESD]. *Genetika* 2003; **39**: 996-1002 [PMID: 12942785]
- 21 **Cox IJ**, Aliev AE, Crossey MM, Dawood M, Al-Mahtab M, Akbar SM, Rahman S, Riva A, Williams R, Taylor-Robinson SD. Urinary nuclear magnetic resonance spectroscopy of a Bangladeshi cohort with hepatitis-B hepatocellular carcinoma: A biomarker corroboration study. *World J Gastroenterol* 2016; **22**: 4191-4200 [PMID: 27122669 DOI: 10.3748/wjg.v22.i16.4191]
- 22 **Robin X**, Turck N, Hainard A, Lisacek F, Sanchez JC, Müller M. Bioinformatics for protein biomarker panel classification: what is needed to bring biomarker panels into in vitro diagnostics? *Expert Rev Proteomics* 2009; **6**: 675-689 [PMID: 19929612 DOI: 10.1586/epr.09.83]
- 23 **Min JH**, Kim YK, Choi SY, Jeong WK, Lee WJ, Ha SY, Ahn S, Ahn HS. Differentiation between cholangiocarcinoma and hepatocellular carcinoma with target sign on diffusion-weighted MR imaging and hepatobiliary phase gadoteric acid-enhanced MR imaging: Classification tree analysis applying capsule and septum. *Eur J Radiol* 2017; **92**: 1-10 [PMID: 28624005 DOI: 10.1016/j.ejrad.2017.04.008]
- 24 **Vohradský J**. Neural network model of gene expression. *FASEB J*

- 2001; **15**: 846-854 [PMID: 11259403 DOI: 10.1096/fj.00-0361com]
- 25 **Wu CF**, Wu YJ, Liang PC, Wu CH, Peng SF, Chiu HW. Disease-free survival assessment by artificial neural networks for hepatocellular carcinoma patients after radiofrequency ablation. *J Formos Med Assoc* 2017; **116**: 765-773 [PMID: 28117199 DOI: 10.1016/j.jfma.2016.12.006]
- 26 **Chen QR**, Vansant G, Oades K, Pickering M, Wei JS, Song YK, Monforte J, Khan J. Diagnosis of the small round blue cell tumors using multiplex polymerase chain reaction. *J Mol Diagn* 2007; **9**: 80-88 [PMID: 17251339 DOI: 10.2353/jmoldx.2007.060111]
- 27 **Nagel MA**, Gilden D, Shade T, Gao B, Cohrs RJ. Rapid and sensitive detection of 68 unique varicella zoster virus gene transcripts in five multiplex reverse transcription-polymerase chain reactions. *J Virol Methods* 2009; **157**: 62-68 [PMID: 19109999 DOI: 10.1016/j.jviromet.2008.11.019]
- 28 **Gerke V**, Creutz CE, Moss SE. Annexins: linking Ca<sup>2+</sup> signalling to membrane dynamics. *Nat Rev Mol Cell Biol* 2005; **6**: 449-461 [PMID: 15928709 DOI: 10.1038/nrm1661]
- 29 **Ahn SH**, Sawada H, Ro JY, Nicolson GL. Differential expression of annexin I in human mammary ductal epithelial cells in normal and benign and malignant breast tissues. *Clin Exp Metastasis* 1997; **15**: 151-156 [PMID: 9062391]
- 30 **Engström W**, Ward A, Moorwood K. The role of scaffold proteins in JNK signalling. *Cell Prolif* 2010; **43**: 56-66 [PMID: 19922489 DOI: 10.1111/j.1365-2184.2009.00654.x]
- 31 **Kanojia D**, Garg M, Gupta S, Gupta A, Suri A. Sperm-associated antigen 9, a novel biomarker for early detection of breast cancer. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 630-639 [PMID: 19190149 DOI: 10.1158/1055-9965.EPI-08-0629]
- 32 **Garg M**, Kanojia D, Salhan S, Suri S, Gupta A, Lohiya NK, Suri A. Sperm-associated antigen 9 is a biomarker for early cervical carcinoma. *Cancer* 2009; **115**: 2671-2683 [PMID: 19326449 DOI: 10.1002/cncr.24293]
- 33 **Shen K**, Xi Z, Xie J, Wang H, Xie C, Lee CS, Fahey P, Dong Q, Xu H. Guttiferone K suppresses cell motility and metastasis of hepatocellular carcinoma by restoring aberrantly reduced profilin 1. *Oncotarget* 2016; **7**: 56650-56663 [PMID: 27494863 DOI: 10.18632/oncotarget.10992]
- 34 **Zou L**, Jaramillo M, Whaley D, Wells A, Panchapakesa V, Das T, Roy P. Profilin-1 is a negative regulator of mammary carcinoma aggressiveness. *Br J Cancer* 2007; **97**: 1361-1371 [PMID: 17940506 DOI: 10.1038/sj.bjc.6604038]
- 35 **Das T**, Bae YH, Wells A, Roy P. Profilin-1 overexpression upregulates PTEN and suppresses AKT activation in breast cancer cells. *J Cell Physiol* 2009; **218**: 436-443 [PMID: 18937284 DOI: 10.1002/jcp.21618]

**P- Reviewer:** Boeckxstaens GE, Lee MW **S- Editor:** Chen K  
**L- Editor:** Wang TQ **E- Editor:** Huang Y





## Retrospective Study

# Clinical advantages of single port laparoscopic hepatectomy

Jae Hyun Han, Young Kyoung You, Ho Joong Choi, Tae Ho Hong, Dong Goo Kim

Jae Hyun Han, Young Kyoung You, Ho Joong Choi, Tae Ho Hong, Dong Goo Kim, Division of Hepatobiliary-Pancreas Surgery and Liver Transplantation, Department of Surgery, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul 06591, South Korea

**Author contributions:** Han JH collected and analyzed the data, and drafted the manuscript; You YK designed and supervised the study; Choi HJ, Hong TH and Kim DG offered the technical and statistical support; all authors have read and approved the final version to be published.

**Institutional review board statement:** This study was reviewed and approved by the Catholic University Seoul St. Mary's Hospital Institutional Review Board.

**Informed consent statement:** Informed consent is exempted in the case of retrospective study in our institution.

**Conflict-of-interest statement:** There are no conflicts of interest to be disclosed.

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**Manuscript source:** Unsolicited manuscript

**Correspondence to:** Young Kyoung You, MD, PhD, Division of Hepatobiliary-Pancreas Surgery and Liver Transplantation, Department of Surgery, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 222, Banpo-daero, Seocho-gu, Seoul 06591, South Korea. [yky602@catholic.ac.kr](mailto:yky602@catholic.ac.kr)  
Telephone: +82-2-22586102  
Fax: +82-2-595-2992

Received: October 10, 2017

Peer-review started: October 10, 2017

First decision: October 30, 2017

Revised: November 9, 2017

Accepted: November 27, 2017

Article in press: November 27, 2017

Published online: January 21, 2018

## Abstract

### AIM

To evaluate the clinical advantages of single-port laparoscopic hepatectomy (SPLH) compare to multi-port laparoscopic hepatectomy (MPLH).

### METHODS

We retrospectively reviewed the medical records of 246 patients who underwent laparoscopic liver resection between January 2008 and December 2015 at our hospital. We divided the surgical technique into two groups; SPLH and MPLH. We performed laparoscopic liver resection for both benign and malignant disease. Major hepatectomy such as right and left hepatectomy was also done with sufficient disease-free margin. The operative time, the volume of blood loss, transfusion rate, and the conversion rate to MPLH or open surgery was evaluated. The post-operative parameters included the meal start date after operation, the number of postoperative days spent in the hospital, and surgical complications was also evaluated.

### RESULTS

Of the 246 patients, 155 patients underwent SPLH and 91 patients underwent MPLH. Conversion rate was 22.6% in SPLH and 19.8% in MPLH ( $P = 0.358$ ). We performed major hepatectomy, which was defined as resection of more than 2 sections, in 13.5% of patients in the SPLH group and in 13.3% of patients in the MPLH group ( $P = 0.962$ ). Mean operative time

was  $136.9 \pm 89.2$  min in the SPLH group and  $231.2 \pm 149.7$  min in the MPLH group ( $P < 0.001$ ). The amount of blood loss was  $385.1 \pm 409.3$  mL in the SPLH group and  $559.9 \pm 624.9$  mL in the MPLH group ( $P = 0.016$ ). The safety resection margin did not show a significant difference ( $0.84 \pm 0.84$  cm in SPLH vs  $1.04 \pm 1.22$  cm in MPLH,  $P = 0.704$ ). Enteral feeding was started earlier in the SPLH group ( $1.06 \pm 0.27$  d after operation) than in the MPLH group ( $1.63 \pm 1.27$  d) ( $P < 0.001$ ). The mean hospital stay after operation was non-significantly shorter in the SPLH group than in the MPLH group ( $7.82 \pm 2.79$  d vs  $7.97 \pm 3.69$  d,  $P = 0.744$ ). The complication rate was not significantly different ( $P = 0.397$ ) and there was no major perioperative complication or mortality case in both groups.

## CONCLUSION

Single-port laparoscopic liver surgery seems to be a feasible approach for various kinds of liver diseases.

**Key words:** Hepatectomy; Laparoscopy; Minimally invasive surgery; Treatment outcome; Feasibility study

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**Core tip:** The progress on the laparoscopic technique has led to single-port laparoscopic surgery as a feasible modality in several abdominal surgeries. However, in the field of liver surgery, single-port surgery have been reported sporadically because of its technical difficulties. In this study, we evaluated the feasibility of single-port laparoscopic hepatectomy (SPLH) compared to multi-port laparoscopic hepatectomy (MPLH). The present study showed that SPLH is not inferior to MPLH in terms of surgical and oncological results. Furthermore, left liver surgery, such as left lateral sectionectomy and left hepatectomy, is possible through single-port without any significant deterioration in results if it is performed by an experienced surgeon.

Han JH, You YK, Choi HJ, Hong TH, Kim DG. Clinical advantages of single port laparoscopic hepatectomy. *World J Gastroenterol* 2018; 24(3): 379-386 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i3/379.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i3.379>

## INTRODUCTION

After introduction of the first laparoscopic surgery, noticeable technical developments have imposed laparoscopic surgery as a valuable alternative to the traditional open surgery<sup>[1,2]</sup>. Since then, the progress on the laparoscopic technique has led to reduced port surgery including single-port laparoscopic surgery as a safe and feasible modality in the field of appendectomy,

cholecystectomy, splenectomy, gastrectomy and colectomy<sup>[3-6]</sup>.

However, in the field of liver surgery, after the first laparoscopic surgery in 1993, complexity of the procedure and technical difficulty are the main causes of delay in its widespread adoption, but its use has steadily and slowly spread in tandem with advances in surgical skill and devices<sup>[7,8]</sup>. In the Louisville Statement 2008, Buell *et al*<sup>[9]</sup> declared that laparoscopic liver surgery is a safe and effective approach for the surgical management of liver disease.

Recently, several investigators have reported that single-port laparoscopic hepatectomy (SPLH) is also a feasible modality like any other single-port laparoscopic surgery<sup>[10-12]</sup>. Nevertheless, almost of these studies are only case reports or small-sized retrospective studies and there is a lack of large clinical randomized trials or systematic reviews that prove its clinical benefits.

The aim of the present study is to investigate the technical feasibility and perioperative results of SPLH compared to the conventional laparoscopic or open surgery in a large volume center.

## MATERIALS AND METHODS

We retrospectively reviewed the medical records of 246 patients who underwent laparoscopic liver resection with curative intent between January 2008 and December 2015 at Seoul St. Mary's Hospital. The patients who underwent concomitant other abdominal surgery were excluded. This study was approved by the Institutional Review Board of our center.

This record was obtained by several experienced hepatobiliary surgeons in our hospital; however, SPLH was performed by one of the surgeons who mainly performed laparoscopic liver resection.

We performed laparoscopic liver resection for both benign and malignant disease. The indications of SPLH and MPLH were not different. Left-lateral sectionectomy and partial hepatectomy for the lesion in the antero-lateral portion of the liver were routinely performed *via* laparoscopy. Major hepatectomy, such as right hepatectomy and left hepatectomy, was also performed *via* laparoscopy if a disease-free margin was expected to be achieved without any major problems. However, the patients with a history of major upper abdominal surgery and cardiac or respiratory impairment were excluded from the laparoscopic approach. Considering the general criteria for liver resection, we excluded the patients with a large amount of ascites or hyperbilirubinemia from liver resection. Patients with Child Pugh class (Child - Turcotte - Pugh) C were also excluded from resection and partial hepatectomy was performed in selected Child Pugh class B patients.

The operative time for each procedure was recorded,

as well as the volume of blood loss, transfusion rate, and the conversion rate to multi-port laparoscopic hepatectomy (MPLH) or open surgery. The post-operative parameters were also recorded, which included the meal start date after operation, the number of postoperative days spent in the hospital, and surgical complications according to the Dindo-Clavien classification<sup>[13]</sup>.

### **Surgical procedure**

Overall, there were few differences between the SPLH and MPLH surgical procedures, except for the difference in trocar use. Generally, the patient was placed in a supine position and for resection of the right liver lobe lesion, the leg was parted. The body of the patient was tilted 10-20° in the head-up and feet-down position. If the lesion was located in the right posterior section, the patient was placed in the right lateral decubitus position at approximately 90°.

For resection of the left liver lobe lesion, the operator stood on the right side of the patient with a scopist, and for right liver lobe resection, the operator stood between the patient's legs with the scopist on the left side of the patient.

For SPLH, a 30 to 40 mm skin incision was made in the right or left upper abdominal quadrant, depending on the location of the liver lesion. Then, Glove port (Nelis, Seoul, South Korea) consisting of four trocar channels with gas insufflation and exsufflation lines was placed. For MPLH, a 10 mm trocar for laparoscopy was inserted into the umbilicus. The 12 mm primary working port was placed below the costal margin depending on the location of the lesion. Then, one or two 5 mm additional working ports were inserted. CO<sub>2</sub> pneumoperitoneum was established at 12 mmHg.

The procedure of laparoscopic hepatectomy was not very different from that in previous studies. We performed intraoperative ultrasonography in almost all cases for the marking of the lesion and hepatic veins. Mobilization of the liver was performed by hook type electrocautery and an ultrasonic scalpel (Harmonic ACE; Ethicon Endo-Surgery, Cincinnati, Ohio, United States) and we generally used a laparoscopic ultrasonic dissector (CUSA; Integra LifeSciences, Plainsboro, NJ, United States) for the deeper part of liver parenchymal dissection and an ultrasonic scalpel for the superficial part of the liver. Small vascular pedicles were sealed with an ultrasonic scalpel, while large vessels and bile ducts were ligated with a metal clip and Hem-o-lok clips (Weck, Research Triangle Park, NC, United States). We generally did not use the argon beam coagulator for coagulation of the resection surface concerning gas embolization.

For retrieval of the specimen, we extended the trocar site in consideration of the specimen volume. In MPLH, we generally extended the trocar site of the umbilicus. A closed suction drain was inserted only

when necessary, in MPLH, a 5mm trocar site was used, and in SPLH, a drain was rarely inserted.

### **Statistical analysis**

Mean, standard deviation, and ranges were used to present numerical variables. Continuous variables were compared by Student's *t*-test. Differences in categorical variables were analyzed with the chi-square test. Logistic regression was used for the multivariate analysis and Cox proportional hazards regression model analysis was used to identify risk factors independently associated with recurrence or survival. The Kaplan-Meier method was used to calculate the disease-free survival and survival rates. The survival time in the groups was compared using the log-rank test. *P*-values < 0.05 were considered to indicate statistical significance.

## **RESULTS**

Of the 246 patients, 155 patients had undergone SPLH, and among them, 120 patients (77.4%) had undergone single-port laparoscopic surgery without open or multiport conversion. In the MPLH group, open conversion was performed in 18 patients (19.8%) and there was no statistically significant difference (*P* = 0.358).

The most common cause of conversion was bleeding in both groups (60% and 33.3%, respectively) and the next most common cause of conversion was adhesion in the MPLH group (22.2%) and technical failure in the SPLH group (14.3%) (Table 1).

The most commonly performed procedure in both groups was partial hepatectomy (65.2% and 39.65%, respectively) and the next most commonly performed procedure was left lateral sectionectomy. Major hepatectomy was performed in 22 cases (14.1%) using a single port and in 12 cases (13.2%) in the MPLH group (Table 2). We did more partial hepatectomy with single-port however, extended cholecystectomy for gallbladder cancer and hepatic cyst marsupialization was done by multi-port.

Tumor distribution was not significantly different between both groups, 11.6% of the SPLH group and 12.1% of the MPLH groups had the tumors over 2 segments (*P* < 0.969).

Patient's demographics was not significantly different between the two groups except BMI. BMI was lower in the MPLH group (*P* < 0.001).

We performed laparoscopic hepatectomy for malignant disease, such as hepatocellular carcinoma and metastatic tumor especially from colon cancer, if we could obtain an adequate safety resection margin. In the SPLH group, 74.8% of patients had malignant disease, and in the MPLH group, the rate of malignant disease was 70.3% (*P* = 0.459).

**Table 1 Cause of conversion *n* (%)**

Cause of conversion	SPLH ( <i>n</i> = 35, 22.6%)	MPLH ( <i>n</i> = 18, 19.8%)
Bleeding	21 (60.0)	6 (33.3)
Adhesion	3 (8.5)	4 (22.2)
Poor localization of tumor	3 (8.5)	2 (11.1)
Advanced tumor	2 (5.7)	1 (5.6)
Technical failure	5 (14.3)	3 (16.7)
Others	1 (3.0)	2 (11.1)

SPLH: Single-port laparoscopic hepatectomy; MPLH: Multi-port laparoscopic hepatectomy.

**Table 2 Comparison of procedure between single-port laparoscopic hepatectomy and multi-port laparoscopic hepatectomy group *n* (%)**

Name of procedure	SPLH ( <i>n</i> = 155, 63.0%)	MPLH ( <i>n</i> = 91, 37.0%)
Right hepatectomy	5 (3.2)	0
Left hepatectomy	17 (10.9)	12 (13.2)
Left lateral sectionectomy	29 (18.7)	19 (20.8)
Segmentectomy	2 (1.3)	3 (3.3)
Partial hepatectomy	101 (65.2)	36 (39.6)
Extended cholecystectomy	0	14 (15.4)
Others	1 (0.7)	7 (7.7)

SPLH: Single-port laparoscopic hepatectomy; MPLH: Multi-port laparoscopic hepatectomy.

We performed single-port laparoscopic major hepatectomy, which was defined as resection of more than 2 sections, in 21 patients (13.5%), and this rate was not significantly different from that in the MPLH group (13.3%,  $P = 0.962$ ).

With respect to operative parameters, we evaluated mean operative time, the amount of transfusion (red blood cell), and safety margin in final pathology reports. Operative time was significantly shorter in the SPLH group ( $P < 0.001$ ). The amount of transfusion was not significantly different between the two groups ( $P = 0.513$ ). The safety resection margin did not show a significant difference between the two groups ( $P = 0.704$ ).

Enteral feeding was started earlier in the SPLH group than in the MPLH group ( $P < 0.001$ ). However, the mean hospital stay after operation was not significantly different between the two groups ( $P = 0.744$ ).

Post-operative complications requiring intervention or surgery occurred at a rate of 7.7% in the SPLH group and at a rate of 3.3% in the MPLH group ( $P = 0.397$ ). However, there was no life-threatening complication or perioperative mortality case in both groups (Table 3).

### Comparative results of left lateral sectionectomy and left hepatectomy (SPLH vs MPLH)

We compared the demographic features and operation-

related factors between the two groups among cases that underwent left lateral sectionectomy and left hepatectomy. Of the 155 patients in the SPLH group, 46 patients (29.7%) underwent left hepatectomy or left lateral sectionectomy, and the number of patients who underwent left hepatectomy or left lateral sectionectomy was 31 (34.1%) in the MPLH group.

Patient's demographics was also not significantly different between the two groups. BMI was not significantly different between the two groups ( $P = 0.337$ ). The rate of liver cirrhosis and the CTP score were also not significantly different between the two groups ( $P = 0.355$  and  $P = 0.106$ , respectively).

The mean operative time was significantly shorter in the SPLH group ( $P < 0.003$ ). The amount of transfusion was not significantly different between the two groups ( $P = 0.513$ ). The safety resection margin also did not show a significant difference between the two groups ( $P = 0.354$ ).

Enteral feeding was also started earlier in the SPLH group than in the MPLH group ( $P < 0.001$ ). However, the mean hospital stay after operation was not significantly different between the two groups ( $P = 0.738$ ).

Post-operative complications requiring intervention or surgery occurred in 4 patients (8.7%) in the SPLH group and in 1 patient (3.2%) in the MPLH group. There was no statistically significant difference (Table 4).

## DISCUSSION

After the first report of single-port laparoscopic surgery in the field of the liver surgery<sup>[10,11]</sup>, several studies have assessed the feasibility of SPLH in selected cases such as left lateral sectionectomy and partial hepatectomy. Most of these studies have reported that SPLH is not inferior to the conventional multiport surgery due to cosmetic advantages, less invasiveness, less hospital duration, and acceptable complication rates like other single-port laparoscopic surgeries such as cholecystectomy and appendectomy<sup>[12,14-17]</sup>.

As previously noted, single-port laparoscopic surgery for the liver has some intrinsic technical limitations<sup>[18,19]</sup>. At first, the loss of triangulation and interference between instruments makes surgery difficult and causes ergonomic problems. Then, the surgical view is relatively narrow because the scope and the instruments are placed in the same line. Thus, if complications develop during surgery such as bleeding or bile leakage during liver resection, it may be difficult to manage with only a single port. In fact, most of the previous studies were regarding left lateral sectionectomy and partial hepatectomy, which are less complex.

In the present study, there is no definite evidence that SPLH could be regarded as an easier technique than MPLH considering the presence of liver cirrhosis and the CTP score. Although, the mean age was lesser



**Table 3** Comparison of results between single-port laparoscopic hepatectomy and multi-port laparoscopic hepatectomy group *n* (%)

Variables	SPLH ( <i>n</i> = 155, 63.0%)	MPLH ( <i>n</i> = 91, 37.0%)	<i>P</i> value
Age	57.1 ± 13.3	60.8 ± 13.4	0.037
Sex (M:F)	105:50	49:42	0.040
BMI	24.1 ± 3.1	22.5 ± 2.9	< 0.001
Liver cirrhosis	51 (32.9)	31 (34.3)	0.887
CTP score	5.37 ± 0.73	5.21 ± 0.64	0.297
Malignant disease	116 (74.8)	64 (70.3)	0.459
Major operation	21 (13.5)	12 (13.3)	0.962
Operation time	136.9 ± 89.2	231.2 ± 149.7	< 0.001
Blood loss (mL)	385.1 ± 409.3	559.9 ± 624.9	0.016
RBC T/F (unit)	0.62 ± 1.98	0.79 ± 1.44	0.513
Conversion rate	35 (22.6)	18 (19.8)	0.358
Enteral feeding (d)	1.06 ± 0.27	1.63 ± 1.27	< 0.001
Hospital stay (d)	7.82 ± 2.79	7.97 ± 3.69	0.744
Disease free margin	0.84 ± 0.84	1.04 ± 1.22	0.704
Complication rate	12 (7.7)	3 (3.3)	0.397
Post op bleeding	1 (8.3)	0	
Pleural effusion	4 (33.3)	1 (33.3)	
Fluid accumulation	6 (50.1)	1 (33.3)	
Others	1 (8.3)	1 (33.3)	

SPLH: Single-port laparoscopic hepatectomy; MPLH: Multi-port laparoscopic hepatectomy; BMI: Body mass index; CTP: Child-Turcotte-Pugh; T/F: Transfusion.

in the SPLH group, the body mass index (BMI) was rather lower in the MPLH group.

Considering surgical results such as the operative time and the mean amount of blood transfusion, the SPLH group seemed to show more favorable results than the MPLH group. However, these commonly unacceptable results can be explained in two ways. The first reason is that the present work was a retrospective study. Although there were no statistically differences between the two groups in terms of patient characteristics, it is possible that SPLH was preferentially applied to cases that appeared slightly easier. The second reason is the surgeon factor. SPLH was initiated at our hospital from 2008 and it has been mostly performed by a single highly experienced liver surgeon. On the other hand, MPLH has been performed by several surgeons with a variety of experience including the one mentioned above. Therefore, it may be inappropriate to compare the two groups; however, it is at least possible to state that SPLH is not very inferior to MPLH considering the surgical results themselves.

The rate of surgical complications showed no significant differences between the two groups. Severe complications such as post-operative bleeding or bile leakage were rare in both groups and the rate of complications that needed surgical or radiologic interventional treatment was comparable to the previously noted results for the conventional open liver surgery<sup>[20,21]</sup>.

This result may be due to a selection bias that there is a possibility that we chose the patients with

less degree of liver cirrhosis for laparoscopic liver surgery. It may also be another reason behind why we preferably selected the cases in which the tumor location is on the antero-lateral surface of the liver as far as possible. However, even on comparing the two laparoscopic hepatectomy groups, the complication rate in the SPLH group was not statistically higher than that in the MPLH group.

We experienced nearly 20% of conversion rate in both groups and it seemed to be higher than previously reported data<sup>[17]</sup> (Aldrighetti, 2012 #220). However, because we preferentially consider laparoscopic surgery and actively adopted it, it will be obviously higher than ordinary cases. What we should be noted is that the conversion rate of both groups was not significantly different.

The length of the hospital stay and the duration of resumption of enteral feeding were shorter in the SPLH group than in the MPLH group. This result has been commonly reported in other previous studies for other single port surgeries<sup>[4,22]</sup>. There are some controversies regarding the claim that single-port laparoscopic surgery needs a shorter recovery period after surgery.

Most of the previous studies for SPLH were performed for benign disease, except for a few studies including our initial reports of SPLH for hepatocellular carcinoma<sup>[12]</sup>. However recently, there have been some controversies regarding the application of MPLH to malignant disease<sup>[23,24]</sup>. Furthermore, the present study also showed that SPLH is not inferior to MPLH in terms of obtaining a sufficient safety resection margin.

The present work includes the results of left lateral

**Table 4 Comparison of results between single-port laparoscopic hepatectomy and multi-port laparoscopic hepatectomy group in left hepatectomy and left lateral sectionectomy *n* (%)**

Variables	SPLH ( <i>n</i> = 46, 59.7%)	MPLH ( <i>n</i> = 31, 40.3%)	<i>P</i> value
Age	59.0 ± 11.1	62.0 ± 9.9	0.223
Sex (M:F)	26:20	16:15	0.816
BMI	23.3 ± 2.8	22.7 ± 2.5	0.337
Liver cirrhosis	9 (19.6)	4 (12.9)	0.525
CTP score	5.44 ± 0.81	5.20 ± 0.56	0.355
Malignant disease	27 (58.7)	12 (38.7)	0.106
Operation time	177.9 ± 114.6	277.6 ± 140.6	0.003
Blood loss (mL)	389.0 ± 270.0	576.9 ± 298.1	0.013
RBC T/F (unit)	0.38 ± 0.9	0.83 ± 0.9	0.094
Conversion rate	15 (32.6)	8 (25.8)	0.616
Enteral feeding (d)	1.08 ± 0.35	1.61 ± 0.89	< 0.001
Hospital stay (d)	9.08 ± 3.21	9.36 ± 3.19	0.738
Disease free margin (cm)	1.17 ± 0.99	1.67 ± 1.92	0.354
Complication rate	4 (8.7)	1 (3.2)	0.402
Post op bleeding	1 (25.0)		
Pleural effusion	0		
Fluid accumulation	2 (50.0)		
Others	1 (25.0)	1 (100)	

SPLH: Single-port laparoscopic hepatectomy; MPLH: Multi-port laparoscopic hepatectomy; BMI: Body mass index; CTP: Child-Turcotte-Pugh; T/F: Transfusion.

sectionectomy, as the previous studies<sup>[15,17]</sup> as well as those of major hepatectomy that involved resection of more than two sections. However, the results of major hepatectomy, especially right hepatectomy showed a largely deviated result in the operative time and the amount of transfusion because of the technical limitations that have been mentioned above. Thus, it is not desirable to compare the results including right hepatectomy and some difficult partial hepatectomy cases that are not suitable for laparoscopic surgery from the beginning. Therefore, we evaluated the results of the left lateral hepatectomy and the left hepatectomy cases that showed the result of even deviations. The results also showed that SPLH is comparable, at least not inferior, to MPLH.

The present study has the limitations of being a retrospective study with a small patient group. However, to the best of our knowledge, it is the first report that adopted SPLH for malignant diseases with compatible results and showed the possibility that it is favorable to apply SPLH for a left liver lobe lesion in terms of surgical and oncologic outcomes.

We have not taken a position that SPLH is superior to MPLH throughout this study. There is an inevitable limitation as it is a retrospective study and the experience of the surgeon who performed SPLH is more than that of another surgeon who performed MPLH.

However, the present study at least showed that SPLH is not inferior to MPLH in terms of surgical and oncological results after favorable patient selection. Furthermore, left liver lobe surgery, such as left lateral sectionectomy and left hepatectomy, is possible

through single-port laparoscopic surgery without any significant deterioration in results compared to MPLH if it is performed by an experienced surgeon.

## ARTICLE HIGHLIGHTS

### Research background

The progress on the laparoscopic technique and instruments has led to single-port laparoscopic surgery as a safe and feasible modality. However, in the field of liver surgery, technical difficulty has delayed its widespread adoption. Recently, several investigators have reported feasible results of single-port laparoscopic hepatectomy (SPLH) however, almost of them are case reports or small sized study.

### Research motivation

Several studies have assessed the feasibility of SPLH in benign diseases such as left lateral sectionectomy and partial hepatectomy in spite of intrinsic technical limitations. However, most of them are for benign diseases and the study size is too small to determine the feasibility.

### Research objectives

The aim of the present study is to investigate the technical feasibility and perioperative results of SPLH compared to the conventional laparoscopic surgery in a large volume center.

### Research methods

Total enrolled patients were 246 and the data was collected from January 2008 to December 2015. The authors divided the surgical technique into two groups; SPLH and multi-port laparoscopic hepatectomy (MPLH). The authors performed laparoscopic liver resection for both benign and malignant disease. Major hepatectomy was done in the case that the disease free margin will be achieved without problems. The operative time, the volume of blood loss, transfusion rate, and the conversion rate to MPLH or open surgery was evaluated. The post-operative parameters included the meal start date after operation, the number of postoperative days spent in the hospital, and surgical complications was also evaluated.

## Research results

In this study, the authors found that the operative results such as the operative time, the volume of blood loss, transfusion rate, and the conversion rate of the SPLH was not inferior to the MPLH. The post-operative parameters such as the meal start date after operation was even better than MPLH. It showed similar results in the analysis of the left liver surgery such as left hepatectomy and left lateral sectionectomy.

## Research conclusions

The present study showed that SPLH is not inferior to MPLH in terms of surgical and oncological results after favorable patient selection. Furthermore, left liver lobe surgery, such as left lateral sectionectomy and left hepatectomy, is possible through single-port laparoscopic surgery without any significant deterioration in results compared to MPLH if it is performed by an experienced surgeon.

## Research perspectives

In the future work, case controlled and/or large size prospective study will be needed.

## REFERENCES

- 1 Antoniou SA, Antoniou GA, Antoniou AI, Granderath FA. Past, Present, and Future of Minimally Invasive Abdominal Surgery. *JSLs* 2015; **19**: pii: e2015.00052 [PMID: 26508823 DOI: 10.4293/JSLs.2015.00052]
- 2 Zhang RC, Zhou YC, Mou YP, Huang CJ, Jin WW, Yan JF, Wang YX, Liao Y. Laparoscopic versus open enucleation for pancreatic neoplasms: clinical outcomes and pancreatic function analysis. *Surg Endosc* 2016; **30**: 2657-2665 [PMID: 26487211 DOI: 10.1007/s00464-015-4538-6]
- 3 Antoniou SA, Koch OO, Antoniou GA, Lasithiotakis K, Chalkiadakis GE, Pointner R, Granderath FA. Meta-analysis of randomized trials on single-incision laparoscopic versus conventional laparoscopic appendectomy. *Am J Surg* 2014; **207**: 613-622 [PMID: 24370108 DOI: 10.1016/j.amjsurg.2013.07.045]
- 4 Antoniou SA, Pointner R, Granderath FA. Single-incision laparoscopic cholecystectomy: a systematic review. *Surg Endosc* 2011; **25**: 367-377 [PMID: 20607556 DOI: 10.1007/s00464-010-1217-5]
- 5 Barbaros U, Dinççağ A. Single incision laparoscopic splenectomy: the first two cases. *J Gastrointest Surg* 2009; **13**: 1520-1523 [PMID: 19365695 DOI: 10.1007/s11605-009-0869-8]
- 6 Takahashi T, Takeuchi H, Kawakubo H, Saikawa Y, Wada N, Kitagawa Y. Single-incision laparoscopic surgery for partial gastrectomy in patients with a gastric submucosal tumor. *Am Surg* 2012; **78**: 447-450 [PMID: 22472403]
- 7 Tzanis D, Shivathirthan N, Laurent A, Abu Hilal M, Soubrane O, Kazaryan AM, Ettore GM, Van Dam RM, Lainas P, Tranchart H, Edwin B, Belli G, Campos RR, Pearce N, Gayet B, Dagher I. European experience of laparoscopic major hepatectomy. *J Hepatobiliary Pancreat Sci* 2013; **20**: 120-124 [PMID: 23053354 DOI: 10.1007/s00534-012-0554-2]
- 8 Pearce NW, Di Fabio F, Teng MJ, Syed S, Primrose JN, Abu Hilal M. Laparoscopic right hepatectomy: a challenging, but feasible, safe and efficient procedure. *Am J Surg* 2011; **202**: e52-e58 [PMID: 21861979 DOI: 10.1016/j.amjsurg.2010.08.032]
- 9 Buell JF, Cherqui D, Geller DA, O'Rourke N, Iannitti D, Dagher I, Koffron AJ, Thomas M, Gayet B, Han HS, Wakabayashi G, Belli G, Kaneko H, Ker CG, Scatton O, Laurent A, Abdalla EK, Chaudhury P, Dutson E, Gamblin C, D'Angelica M, Nagorney D, Testa G, Labow D, Manas D, Poon RT, Nelson H, Martin R, Clary B, Pinson WC, Martinie J, Vauthey JN, Goldstein R, Roayaie S, Barlet D, Espat J, Abecassis M, Rees M, Fong Y, McMasters KM, Broelsch C, Busuttil R, Belghiti J, Strasberg S, Chari RS; World Consensus Conference on Laparoscopic Surgery. The international position on laparoscopic liver surgery: The Louisville Statement, 2008. *Ann Surg* 2009; **250**: 825-830 [PMID: 19916210]
- 10 Gaujoux S, Kingham TP, Jarnagin WR, D'Angelica MI, Allen PJ, Fong Y. Single-incision laparoscopic liver resection. *Surg Endosc* 2011; **25**: 1489-1494 [PMID: 20976489 DOI: 10.1007/s00464-010-1419-x]
- 11 Chang SK, Mayasari M, Ganpathi IS, Wen VL, Madhavan K. Single port laparoscopic liver resection for hepatocellular carcinoma: a preliminary report. *Int J Hepatol* 2011; **2011**: 579203 [PMID: 21994864 DOI: 10.4061/2011/579203]
- 12 Shetty GS, You YK, Choi HJ, Na GH, Hong TH, Kim DG. Extending the limitations of liver surgery: outcomes of initial human experience in a high-volume center performing single-port laparoscopic liver resection for hepatocellular carcinoma. *Surg Endosc* 2012; **26**: 1602-1608 [PMID: 22179464 DOI: 10.1007/s00464-011-2077-3]
- 13 Clavien PA, Barkun J, de Oliveira ML, Vauthey JN, Dindo D, Schulick RD, de Santibañes E, Pekolj J, Slankamenac K, Bassi C, Graf R, Vonlanthen R, Padbury R, Cameron JL, Makuuchi M. The Clavien-Dindo classification of surgical complications: five-year experience. *Ann Surg* 2009; **250**: 187-196 [PMID: 19638912 DOI: 10.1097/SLA.0b013e3181b13ca2]
- 14 Tayar C, Subar D, Salloum C, Malek A, Laurent A, Azoulay D. Single incision laparoscopic hepatectomy: Advances in laparoscopic liver surgery. *J Minim Access Surg* 2014; **10**: 14-17 [PMID: 24501503 DOI: 10.4103/0972-9941.124454]
- 15 Hu M, Zhao G, Wang F, Xu D, Liu R. Single-port and multi-port laparoscopic left lateral liver sectionectomy for treating benign liver diseases: a prospective, randomized, controlled study. *World J Surg* 2014; **38**: 2668-2673 [PMID: 24867469 DOI: 10.1007/s00268-014-2610-3]
- 16 Pan M, Jiang Z, Cheng Y, Xu X, Zhang Z, Zhou C, He G, Xu T, Liu H, Gao Y. Single-incision laparoscopic hepatectomy for benign and malignant hepatopathy: initial experience in 8 Chinese patients. *Surg Innov* 2012; **19**: 446-451 [PMID: 22474017 DOI: 10.1177/1553350612438412]
- 17 Aldrighetti L, Ratti F, Catena M, Pulitanò C, Ferla F, Cipriani F, Ferla G. Laparoendoscopic single site (LESS) surgery for left-lateral hepatic sectionectomy as an alternative to traditional laparoscopy: case-matched analysis from a single center. *Surg Endosc* 2012; **26**: 2016-2022 [PMID: 22278101 DOI: 10.1007/s00464-012-2147-1]
- 18 Lirici MM. Single site laparoscopic surgery: an intermediate step toward no (visible) scar surgery or the next gold standard in minimally invasive surgery? *Minim Invasive Ther Allied Technol* 2012; **21**: 1-7 [PMID: 22049942 DOI: 10.3109/13645706.2011.631551]
- 19 Islam A, Castellvi AO, Tesfay ST, Castellvi AD, Wright AS, Scott DJ. Early surgeon impressions and technical difficulty associated with laparoendoscopic single-site surgery: a Society of American Gastrointestinal and Endoscopic Surgeons Learning Center study. *Surg Endosc* 2011; **25**: 2597-2603 [PMID: 21359887 DOI: 10.1007/s00464-011-1594-4]
- 20 Benzon E, Cojutti A, Lorenzin D, Adani GL, Baccarani U, Favero A, Zompicchiati A, Bresadola F, Uzzau A. Liver resective surgery: a multivariate analysis of postoperative outcome and complication. *Langenbecks Arch Surg* 2007; **392**: 45-54 [PMID: 16983576 DOI: 10.1007/s00423-006-0084-y]
- 21 Asiyabola B, Chang D, Gleisner AL, Nathan H, Choti MA, Schulick RD, Pawlik TM. Operative mortality after hepatic resection: are literature-based rates broadly applicable? *J Gastrointest Surg* 2008; **12**: 842-851 [PMID: 18266046 DOI: 10.1007/s11605-008-0494-y]
- 22 Kim HO, Yoo CH, Lee SR, Son BH, Park YL, Shin JH, Kim H, Han WK. Pain after laparoscopic appendectomy: a comparison of transumbilical single-port and conventional laparoscopic surgery. *J Korean Surg Soc* 2012; **82**: 172-178 [PMID: 22403751 DOI: 10.4174/jkss.2012.82.3.172]
- 23 Yoon YI, Kim KH, Kang SH, Kim WJ, Shin MH, Lee SK, Jung

DH, Park GC, Ahn CS, Moon DB, Ha TY, Song GW, Hwang S, Lee SG. Pure Laparoscopic Versus Open Right Hepatectomy for Hepatocellular Carcinoma in Patients With Cirrhosis: A Propensity Score Matched Analysis. *Ann Surg* 2017; **265**: 856-863 [PMID: 27849661 DOI: 10.1097/SLA.0000000000002072]

24 **Komatsu S**, Brustia R, Goumard C, Perdigao F, Soubrane O, Scatton O. Laparoscopic versus open major hepatectomy for hepatocellular carcinoma: a matched pair analysis. *Surg Endosc* 2016; **30**: 1965-1974 [PMID: 26194255 DOI: 10.1007/s00464-015-4422-4]

**P- Reviewer:** Fabozzi M, Mastoraki A, Memeo R, Noda H  
**S- Editor:** Ma YJ **L- Editor:** A **E- Editor:** Huang Y





## Retrospective Study

# Autoimmune liver disease-related autoantibodies in patients with biliary atresia

Shu-Yin Pang, Yu-Mei Dai, Rui-Zhong Zhang, Yi-Hao Chen, Xiao-Fang Peng, Jie Fu, Zheng-Rong Chen, Yun-Feng Liu, Li-Yuan Yang, Zhe Wen, Jia-Kang Yu, Hai-Ying Liu

Shu-Yin Pang, Yu-Mei Dai, Yi-Hao Chen, Yun-Feng Liu, Li-Yuan Yang, Hai-Ying Liu, Clinical Laboratory, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou 510623, Guangdong Province, China

Rui-Zhong Zhang, Xiao-Fang Peng, Jie Fu, Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou 510623, Guangdong Province, China

Zheng-Rong Chen, Department of Pathology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou 510623, Guangdong Province, China

Zhe Wen, Jia-Kang Yu, Department of Neonatal Surgery, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou 510623, Guangdong Province, China

ORCID number: Shu-Yin Pang (0000-0002-8193-9866); Yu-Mei Dai (0000-0003-4157-8653); Rui-Zhong Zhang (0000-0002-4954-7192); Yi-Hao Chen (0000-0002-4670-3532); Xiao-Fang Peng (0000-0001-7669-3136); Jie Fu (0000-0002-1700-6594); Zheng-Rong Chen (0000-0002-7344-5452); Yun-Feng Liu (0000-0001-5801-7655); Li-Yuan Yang (0000-0002-0837-9292); Zhe Wen (0000-0002-6062-1242); Jia-Kang Yu (0000-0002-7634-3275); Hai-Ying Liu (0000-0001-5680-3492).

**Author contributions:** Pang SY, Chen YH, Peng XF and Fu J performed the majority of experiments; Chen ZR reviewed the liver sections; Liu YF and Yang LY analyzed the data and contributed to editing of the manuscript; Wen Z and Yu JK collected all the clinical information; Pang SY, Dai YM, Zhang RZ and Liu HY designed the study and wrote the manuscript.

**Supported by the Guangdong Provincial Science and Technology Planning Project, No. 2014A020212520; and the Guangzhou Science and Technology Project, No. 201707010014.**

**Institutional review board statement:** The study protocol was approved by the Ethics Committee of Guangzhou Women and Children's Medical Center, No. 2015090117.

**Informed consent statement:** Informed consent was obtained from the legal guardian of all patients prior to study enrolment.

**Conflict-of-interest statement:** The authors declare no conflict of interests related to this study.

**Data sharing statement:** No additional data are available.

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**Manuscript source:** Unsolicited manuscript

**Correspondence to:** Hai-Ying Liu, PhD, Chief Technician, Clinical Laboratory, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, No. 9, Jinsui Road, Guangzhou 510623, Guangdong Province, China. [xiangliu haiying@aliyun.com](mailto:xiangliu haiying@aliyun.com)  
Telephone: +86-20-38076255  
Fax: +86-20-38076255

**Received:** November 9, 2017  
**Peer-review started:** November 9, 2017  
**First decision:** November 30, 2017  
**Revised:** December 14, 2017  
**Accepted:** December 20, 2017  
**Article in press:** December 20, 2017  
**Published online:** January 21, 2018

## Abstract

### AIM

To investigate the prevalence and clinical significance of

autoimmune liver disease (ALD)-related autoantibodies in patients with biliary atresia (BA).

# METHODS

Sera of 124 BA patients and 140 age-matched non-BA controls were assayed for detection of the following autoantibodies: ALD profile and specific anti-nuclear antibodies (ANAs), by line-blot assay; ANA and anti-neutrophil cytoplasmic antibody (ANCA), by indirect immunofluorescence assay; specific ANCAs and anti-M2-3E, by enzyme linked immunosorbent assay. Associations of these autoantibodies with the clinical features of BA (*i.e.*, cytomegalovirus infection, degree of liver fibrosis, and short-term prognosis of Kasai procedure) were evaluated by Spearman's correlation coefficient.

# RESULTS

The overall positive rate of serum autoantibodies in preoperative BA patients was 56.5%. ALD profile assay showed that the positive reaction to primary biliary cholangitis-related autoantibodies in BA patients was higher than that to autoimmune hepatitis-related autoantibodies. Among these autoantibodies, anti-BPO was detected more frequently in the BA patients than in the controls (14.8% *vs* 2.2%,  $P < 0.05$ ). Accordingly, 32 (25.8%) of the 124 BA patients also showed a high positive reaction for anti-M2-3E. By comparison, the controls had a remarkably lower frequency of anti-M2-3E ( $P < 0.05$ ), with 6/92 (8.6%) of patients with other liver diseases and 2/48 (4.2%) of healthy controls. The prevalence of ANA in BA patients was 11.3%, which was higher than that in disease controls (3.3%,  $P < 0.05$ ), but the reactivity to specific ANAs was only 8.2%. The prevalence of ANCAs (ANCA or specific ANCAs) in BA patients was also remarkably higher than that in the healthy controls (37.9% *vs* 6.3%,  $P < 0.05$ ), but showed no difference from that in patients with other cholestasis. ANCA positivity was closely associated with the occurrence of postoperative cholangitis ( $r = 0.61$ ,  $P < 0.05$ ), whereas none of the autoantibodies showed a correlation to cytomegalovirus infection or the stages of liver fibrosis.

# CONCLUSION

High prevalence of autoantibodies in the BA developmental process strongly reveals the autoimmune-mediated pathogenesis. Serological ANCA positivity may be a useful predictive biomarker of postoperative cholangitis.

**Key words:** Biliary atresia; Anti-nuclear antibody; Anti-neutrophilic cytoplasmic antibody; Autoimmune liver diseases; Autoantibodies

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**Core tip:** The autoimmune-mediated pathogenesis of biliary atresia (BA) is not fully understood, and non-invasive diagnostic methods cannot clearly discriminate

BA from other causes of neonatal cholestasis. We investigated the prevalence and clinical significance of autoimmune liver disease-related autoantibodies in BA patients. The overall positive rate of autoantibodies in BA was 56.5%. The data showed that frequent detection of autoantibodies in BA may strongly support the autoimmune-mediated pathogenesis. Interestingly, preoperative anti-neutrophil cytoplasmic antibody positivity was closely associated with prediction of cholangitis occurrence after Kasai portoenterostomy.

Pang SY, Dai YM, Zhang RZ, Chen YH, Peng XF, Fu J, Chen ZR, Liu YF, Yang LY, Wen Z, Yu JK, Liu HY. Autoimmune liver disease-related autoantibodies in patients with biliary atresia. *World J Gastroenterol* 2018; 24(3): 387-396 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i3/387.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i3.387>

# INTRODUCTION

Biliary atresia (BA) is a severe neonatal disease, characterized by progressive inflammatory fibrosis and obliteration of both the intra-hepatic and extra-hepatic bile ducts<sup>[1,2]</sup>. Early Kasai portoenterostomy (KP), the first-line treatment for BA, may re-establish bile flow to alleviate liver injury caused by cholestasis and to prolong survival with the native liver<sup>[3]</sup>. However, in the majority of BA patients, the continued existence of the bile duct injury may eventually lead to cirrhosis and need for liver transplantation<sup>[4,5]</sup>. Thus, gaining a better understanding of the pathogenic mechanisms underlying BA may facilitate early diagnosis or development of clinical therapies to halt the injury of hepatic bile ducts and to preserve liver function.

Although the etiology of BA is not fully understood, accumulated evidence in the literature supports the theory that a primary perinatal viral infection triggers an aberrant autoimmune-mediated attack on bile duct epithelia by molecular mimicry, with both the cellular and humoral immunity playing important roles in the BA autoimmune injury mechanism<sup>[6-9]</sup>. Several viruses have been proposed as the infectious agents, and perinatal infection with the cytomegalovirus (CMV) has been demonstrated as an important etiological factor for BA in China<sup>[10]</sup>. Periductal immunoglobulin (Ig) and circulating autoantibodies which might be used in the classification of autoimmune diseases have been described in both patients with BA and animal models of the disease<sup>[11,12]</sup>; unfortunately, the specificity of these autoantibodies for BA has been far less satisfying.

BA and autoimmune liver disease (ALD) have some similar clinical manifestations and pathological features. However, ALD-related autoantibodies have not yet been comprehensively investigated in BA patients, to the best of our knowledge. Here, we describe our investigation into the prevalence of the ALD profile and the extent of positivity of anti-nuclear antibodies (ANAs)

**Table 1** Demographic and clinical features, and biochemical parameters<sup>1</sup> of biliary atresia patients and non-biliary atresia controls *n* (%)

Variable	BA, <i>n</i> = 124	Non-BA, <i>n</i> = 140	
		Other liver diseases <sup>2</sup> , <i>n</i> = 92	Healthy, <i>n</i> = 48
Age, mo	2.9 (1.9-3.0)	2.8 (2.0-3.2)	3.4 (2.0-4.0)
Sex, male/female	60/64	50/42	25/23
METAVIR score			
F0	4 (3.2)	NA	NA
F1	37 (29.8)	NA	NA
F2	24 (19.4)	NA	NA
F3	50 (40.3)	NA	NA
F4	9 (7.3)	NA	NA
ALT, U/L	162.4 (104.3-204.6) <sup>a</sup>	132.0 (70.5-247.5)	28.1 (22.4-40.8)
AST, U/L	190.5 (151.4-257.4) <sup>a</sup>	195.9 (117.0-292.5)	38.0 (26.0-52.3)
γ-GT, U/L	716.0 (411.0-1142.5) <sup>a</sup>	598.4 (189.5-1043.2)	32.7 (23.4-46.3)
ALP, U/L	406.8 (316.5-522.2) <sup>a</sup>	517.0 (409.7-660.3)	275.8 (189.0-345.6)
TBIL, μmol/L	157.0 (126.5-184.0) <sup>a</sup>	145.7 (107.1-196.5)	6.5 (2.8-14.8)
DBIL, μmol/L	130.5 (103.5-152.7) <sup>a</sup>	110.1 (88.4-137.8)	3.1 (1.0-4.9)

Data are described as median (interquartile range: 25<sup>th</sup>-75<sup>th</sup> percentile). <sup>1</sup>Reference intervals: ALT, 3-35 U/L; AST, 5-60 U/L; γ-GT, 13-57 U/L; ALP, 118-390 U/L; TBIL, 2-17 μmol/L; DBIL, 0-7 μmol/L; <sup>2</sup>Choledochal cysts, transient cholestasis of unknown origin, and neonatal intrahepatic cholestasis caused by citrin deficiency were included as disease controls. <sup>a</sup>*P* < 0.05 *vs* healthy controls. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BA: Biliary atresia; DBIL: Direct bilirubin; FO-F4: Fibrosis scores 0-4; γ-GT: Gamma-glutamyl transpeptidase; NA: Not applicable; TBIL: Total bilirubin.

and anti-neutrophilic cytoplasmic antibodies (ANCAs) in the sera of BA patients. The associations of these autoantibodies with the clinical features of BA were also assessed statistically.

## MATERIALS AND METHODS

### Case enrollment

A total of 124 preoperative BA patients [mean age: 2.9 mo (interquartile range, IQR: 1.9-3.0)], 92 controls with other liver diseases [mean age: 2.8 mo (IQR: 2.0-3.2); including 42 with choledochal cysts, 35 with transient cholestasis of unknown origin, and 15 with neonatal intrahepatic cholestasis caused by citrin deficiency], and 48 healthy controls [mean age: 3.4 mo (IQR: 2.0-4.0)] were enrolled in this study. Table 1 shows the demographic and clinical features, and biochemical parameters of the study population. All study participants originated from Guangzhou Women and Children's Medical Center (Guangzhou, China) between January 2015 and December 2016. Enrollment was proposed to all consecutive infants with diagnosed BA that had been confirmed by surgical exploration, cholangiography and histology. Diagnosis of all controls was based on the criteria published in our previous report<sup>[13]</sup>. Clinical information was collected when available, including CMV infection, biochemical indexes, histological liver fibrosis stages, and short-term outcomes. Histological liver fibrosis in BA was assessed by METAVIR fibrosis scores (F0-F4)<sup>[14,15]</sup>. Follow-up data which could evaluate persistence of jaundice (total bilirubin, TB: > 34 μmol/L), acute liver injury (alanine aminotransferase, ALT: >35 U/L), and occurrence of cholangitis within 3-10 mo after KP were

collected for analysis of short-term outcomes<sup>[16]</sup>.

### ALD profile

The line-blot ALD profile contains the primary biliary cholangitis (PBC)-related antibodies [anti-mitochondrial antibody, AMA-M2 (pyruvate dehydrogenase complex, PDC), anti-BPO (recombinant fusion proteins of the E2 subunits derived from the 2-oxo-acid dehydrogenase complex targeted by the inner mitochondrial membrane), anti-Sp100, anti-promyelocytic leukemia protein (PML), and anti-gp210], autoimmune hepatitis (AIH)-related antibodies [anti-liver-kidney microsomal type 1 (LKM-1), anti-liver cytosolic antigen type 1 (LC-1), and anti-soluble liver antigen/liver-pancreas (SLA/LP)], and anti-Ro-52 antibodies. A commercially available kit (EUROIMMUN AG, Lübeck, Germany) was used according to the manufacturer's instructions. Signal strengths of > 10 arbitrary units (AUs) were considered positive.

AMA reactivity was confirmed, with enlarged sample size by an enhanced anti-M2-3E enzyme linked immunosorbent assay (ELISA), which mixed enveloped recombinant fusion protein BPO and natively purified PDC from bovine heart mitochondria as antigenic targets (EUROIMMUN AG).

### ANA and specific ANAs

ANA was detected by indirect immunofluorescence (IIF) using the antigen substrate panel of Hep-2 cells and primate liver. A serum titer ≥ 1:100 was considered positive. ANA positivity was subgrouped based upon the specific fluorescence patterns. Accordingly, line-blot immunoassay was used to determine the IgG autoantibody panel for 12 specific ANAs, which

**Table 2** Prevalence profile of autoimmune liver disease in biliary atresia patients and non-biliary atresia controls *n* (%)

Variable	BA, <i>n</i> = 81	Non-BA, <i>n</i> = 88		
		Other liver diseases <sup>1</sup> , <i>n</i> = 44	Healthy, <i>n</i> = 40	Total
PBC-related antibodies	15 (18.5) <sup>c</sup>	4 (9.1)	1 (2.5)	5 (5.7)
AMA-M2	1 (1.2)	0 (0)	0 (0)	0 (0)
Anti-BPO	12 (14.8) <sup>a,c</sup>	2 (4.5)	0 (0)	2 (2.2)
Anti-Sp100	1 (1.2)	0 (0)	0 (0)	0 (0)
Anti-gp210	2 (2.5)	1 (2.3)	1 (2.5)	2 (2.2)
Anti-PML	3 (3.7)	1 (2.3)	0 (0)	1 (1.1)
AMA-M2 + anti-BPO	1 (1.2)	0 (0)	0 (0)	0 (0)
AMA-M2 + anti-BPO + anti-Sp100 + anti-PML	1 (1.2)	0 (0)	0 (0)	0 (0)
AIH-related antibodies	6 (7.4)	3 (6.8)	3 (7.5)	6 (6.8)
Anti-LKM-1	0 (0)	0 (0)	0 (0)	0 (0)
Anti-LC-1	5 (6.2)	3 (6.8)	3 (7.5)	6 (6.8)
Anti-SLA/LP	1 (1.2)	0 (0)	0 (0)	0 (0)
Anti-Ro-52	5 (6.2)	2 (4.5)	2 (5)	4 (4.5)

<sup>1</sup>Cholelithal cysts, transient cholestasis of unknown origin, and neonatal intrahepatic cholestasis caused by citrin deficiency were included as disease controls. AMA-M2 + M2-3E: combined the positivity to AMA-M2 and BPO; AMA-M2 + BPO + Sp100 + PML: Combined the positivity to AMA-M2, BPO, Sp100, and PML. <sup>a</sup>*P* < 0.05 *vs* non-BA; <sup>c</sup>*P* < 0.05 *vs* healthy controls. AIH: Autoimmune hepatitis; BA: Biliary atresia; PBC: Primary biliary cholangitis; LKM-1: Liver-kidney microsomal type 1; LC-1: Liver cytosolic antigen type 1.

consisted of anti-nRNP/Sm, anti-Sm, anti-SS-A, anti-Ro-52, anti-SS-B, anti-Scl-70, anti-Jo-1, anti-CENP B, anti-dsDNA, anti-nucleosomes, anti-histone, and anti-ribosomal phosphoprotein. Experiments were performed following the manufacturer's instructions (EUROIMMUN AG).

### ANCA and specific ANCAs

A commercially available IIF assay was used for determination of ANCA on ethanol- and formaldehyde-fixed human neutrophils (EUROIMMUN AG). A positive ANCA finding was defined as a titer of antibodies > 1:10. The ANCA findings were subgrouped into cytoplasmic (c)-ANCA, perinuclear (p)-ANCA, and atypical (a)-ANCA according to the fluorescence patterns. Specific ANCAs of myeloperoxidase (MPO) and proteinase 3 (PR3) were further assayed by ELISA (EUROIMMUN AG).

### Association of autoantibodies with clinical features

To determine whether the presence of autoantibodies in BA patients was associated with worse disease progression, we compared the clinical features of the BA patients who presented with and without autoantibodies. The clinical features of 124 BA patients (mainly composed of those with CMV infection) and degree of liver fibrosis were retrospectively analyzed for the period prior to the KP; in addition, the information of short-term outcomes in 52 BA patients who were followed postoperatively for > 3 mo was collected, and 24 of those 52 cases were re-assessed for preoperative and postoperative serum autoantibodies to compare the change of autoantibodies over time.

### Statistical analysis

Normally distributed variables are represented as mean ± SD, and non-normally distributed variables as median (IQR). Categorical data are described

as frequencies and/or percentages. For continuous variables, between-group differences were compared using the Student's *t*-test or the Mann-Whitney *U* test. For categorical variables, the  $\chi^2$  test or Fisher's exact test was used to compare the prevalence between groups when appropriate. Correlation was evaluated by the Spearman's correlation coefficient. SPSS 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Armonk, NY, United States) was used to perform all statistical analyses. *P* values < 0.05 were considered statistically significant.

## RESULTS

### ALD profile in patients with BA compared to controls

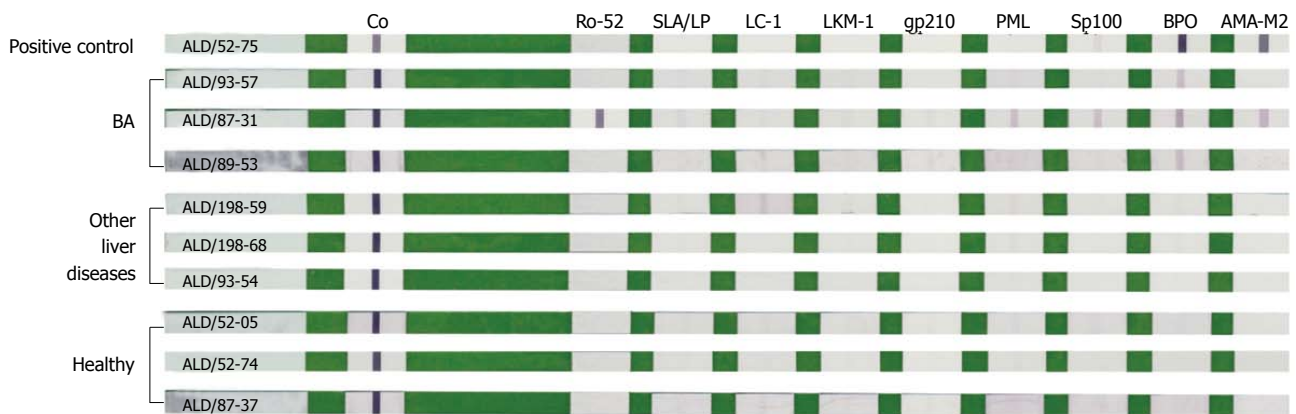
Sera of 81 postoperative BA patients and 88 non-BA controls were evaluated for ALD profile (Table 2). The 88 non-BA controls consisted of 40 healthy controls and 48 disease controls, including 22 with choledochal cysts, 14 with neonatal intrahepatic cholestasis caused by citrin deficiency, and 12 with transient cholestasis of unknown origin. One or more of the PBC-related antibodies was detected in 18.5% (15/81) of the patients with BA. For the PBC-related antibodies, a positive reaction to AMA-M2, anti-BPO, anti-Sp100, anti-gp210, and anti-PML in BA patients was found in 1.2%, 14.8%, 1.2%, 2.5% and 3.7%, respectively. Among these autoantibodies, anti-BPO was detected more frequently in the BA patients than in the non-BA controls (14.8% *vs* 2.2%, *P* < 0.05) or the healthy controls (14.8% *vs* 0%, *P* < 0.05). The prevalence of any other antibodies was not different between the BA and non-BA groups. Only 1 (1.2%) in 81 of the BA patients showed positivity for both AMA-M2 and anti-BPO (Figure 1). For the AIH-related antibodies, low positivity was found in both the BA patients and non-BA controls (7.4% and 6.8%, respectively, *P* > 0.05). Among the total 81 postoperative BA patients, 5 (6.2%)



**Table 3** Prevalence of anti-nuclear antibodies in biliary atresia patients and non-biliary atresia controls *n* (%)

Variable	BA, <i>n</i> = 124	Other liver diseases <sup>1</sup> , <i>n</i> = 92	Healthy, <i>n</i> = 48
ANA, by IIF	14 (11.3) <sup>c</sup>	3 (3.3)	2 (4.2)
Fluorescence patterns			
Homogeneous	3 (2.4)	0 (0)	0 (0)
Speckled	3 (2.4)	0 (0)	1 (2.1)
Nucleolar	3 (2.4)	0 (0)	1 (2.1)
Nuclear dots	1 (0.8)	0 (0)	0 (0)
Centrosome	1 (0.8)	0 (0)	0 (0)
Cytoplasm	1 (0.8)	0 (0)	0 (0)
Ring or rod Golgi	1 (0.8)	2 (2.2)	0 (0)
Centromere	1 (0.8)	0 (0)	0 (0)
Spindle apparatus	0 (0)	1 (1.1)	0 (0)
Specific ANA <sup>2</sup> , by line-blot			
SSA	1 (0.8)	0 (0)	0 (0)
Ro-52	5 (4.0)	2 (2.2)	2 (4.2)
CENP B	4 (3.2)	1 (1.1)	0 (0)
dsDNA	3 (2.4)	0 (0)	0 (0)

<sup>1</sup>Cholelithiasis, transient cholestasis of unknown origin, and neonatal intrahepatic cholestasis caused by citrin deficiency were included as disease controls; <sup>2</sup>Specific ANAs included 12 different antibodies, with only SSA, Ro-52, CENP B, and dsDNA positive in the study. <sup>c</sup>*P* < 0.05 vs other liver diseases. ANA: Anti-nuclear antibody; BA: Biliary atresia; IIF: Indirect immunofluorescence.



**Figure 1** Representative strips after color development by line-blot immunoassay. The line-blot immunoassay strips had been coated with nine autoimmune liver disease-related antigens, including Ro-52, SLA/LP, LC-1, LKM-1, gp210, PML, Sp100, BPO and AMA-M2 (from left to right). BA group: ALD/93-57 with anti-BPO +; ALD/87-31 with anti-Ro-52 +++, anti-PML +, anti-Sp100 +, anti-BPO ++, and AMA-M2 +; ALD/89-53 with anti-BPO +; Other liver diseases group: Only ALD/198-59 with anti-LC-1 ±; Healthy group: All autoantibodies were negative. Positive control (ALD/52-75) showed anti-BPO +++ and AMA-M2 +++. Other liver diseases include choledochal cysts, transient cholestasis of unknown origin, and neonatal intrahepatic cholestasis caused by citrin deficiency. ALD: Autoimmune liver disease; BA: Biliary atresia; LC-1: Liver cytosolic antigen type 1.

and 1 (1.2%) showed positivity for anti-LC-1 and anti-SLA/LP, respectively.

With enlarged sample size, AMA reactivity was confirmed by an enhanced anti-M2-3E ELISA. As presented in Figure 2, 32 of the 124 BA patients showed a higher positive reaction to anti-M2-3E compared to the disease controls and the healthy controls (25.8% vs 8.6%, *P* < 0.05 and 25.8% vs 4.2%, *P* < 0.05, respectively).

#### ANA and specific ANAs in patients with BA compared to controls

ANA exhibited a higher prevalence in 124 BA patients compared to 92 patients with other liver diseases (11.3% vs 3.3%, *P* < 0.05), but showed no difference from that in 48 healthy controls (4.2%). Nuclear homogeneous, speckled, and nucleolar types were

the main fluorescence patterns of ANA positivity in BA (Table 3). Patterns involving nuclear dots, centrosome, cytoplasm, ring or rod Golgi, and centromere were present occasionally.

The specific ANA findings in the BA patients evaluated in this study were as follows (Table 3): positivity for anti-SSA in 1, for anti-Ro-52 in 5, for anti-CENP B in 4, and for anti-dsDNA in 3. At least one autoantibody was present in 8.9% (11/124) of the BA patients, which was a higher rate compared to the non-BA controls, but the difference did not reach the threshold for statistical significance. No other specific ANAs were found in the sera of BA patients.

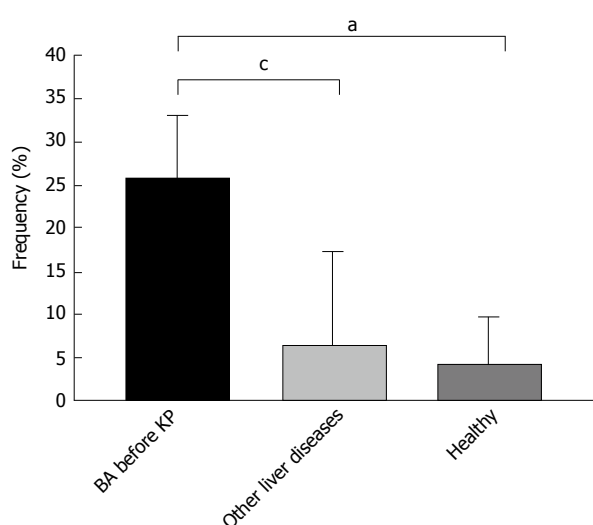
#### ANCA and specific ANCAs in patients with BA compared to controls

Twenty-nine percent (36/124) of BA patients were

**Table 4** Prevalence of anti-neutrophil cytoplasmic antibodies in biliary atresia patients and non-biliary atresia controls *n* (%)

Variable	BA, <i>n</i> = 124	Other liver diseases <sup>1</sup> , <i>n</i> = 92	Healthy, <i>n</i> = 48
ANCA, by IIF	36 (29.0) <sup>a</sup>	23 (25.0)	2 (4.2)
c-ANCA	10 (8.1)	1 (1.1)	0 (0)
p-ANCA	25 (20.2)	21 (22.8)	2 (4.2)
a-ANCA	1 (0.8)	1 (1.1)	0 (0)
Specific ANCA, by ELISA			
Anti-MPO	11 (8.9)	6 (6.5)	2 (4.2)
Anti-PR3	19 (15.3)	13 (14.2)	0 (0)
Anti-MPO and/or anti-PR3	23 (18.0) <sup>a</sup>	13 (14.2)	2 (4.2)
ANCAs	47 (37.9) <sup>a</sup>	31 (33.7)	3 (6.3)

<sup>1</sup>Choledochal cysts, transient cholestasis of unknown origin, and neonatal intrahepatic cholestasis caused by citrin deficiency were included as disease controls. <sup>a</sup>*P* < 0.05 vs healthy controls. Anti-MPO and/or anti-PR3: Any positivity of specific ANCAs; ANCAs: Any positivity of ANCAs by IIF or specific ANCA by ELISA. ANCA: Anti-neutrophil cytoplasmic antibody; BA: Biliary atresia; ELISA: Enzyme linked immunosorbent assay; IIF: Indirect immunofluorescence.



**Figure 2** Positivity of anti-M2-3E detected by enzyme linked immunosorbent assay in biliary atresia patients and controls. <sup>a</sup>*P* < 0.05 vs healthy controls; <sup>c</sup>*P* < 0.05 vs other liver diseases. Other liver diseases include choledochal cysts, transient cholestasis of unknown origin, and neonatal intrahepatic cholestasis caused by citrin deficiency. BA: Biliary atresia; ELISA: Enzyme linked immunosorbent assay.

positive for ANCA. The prevalence was similar to that of the patients with other liver diseases (25.0%, *P* > 0.05), and was higher than that of the healthy controls (4.2%, *P* < 0.05) (Table 4). As shown in Figure 3, p-ANCA and c-ANCA were present more frequently than a-ANCA in BA patients (20.2% and 8.1% compared to 0.8%, respectively)

Anti-MPO and anti-PR3, alone or associated, were found in 23/124 (18.0%) of BA patients, whose positive rates were higher than those in the healthy controls (*P* < 0.05). Among the 124 BA patients, 7 showed positive reactions for both anti-MPO and anti-PR3. The prevalence of ANCAs (ANCA, anti-MPO, or anti-PR3) in BA patients was higher than that in healthy controls (37.9% vs 6.3%, *P* < 0.05).

#### Association of autoantibodies with clinical features

In this study, anti-M2-3E, ANA, and ANCA had higher

prevalence in 124 BA patients than in 140 non-BA controls. Among the BA patients, 56.5% showed positivity for at least one type of the autoantibodies (anti-M2-3E, ANA, and ANCA). Among the 80 BA patients, 45% had a perinatal infection with CMV (detected by combined analysis of anti-CMV IgG, IgM, and CMV DNA). The percentage of CMV infection in the anti-M2-3E-positive BA patients was similar to that in the anti-M2-3E-negative BA patients (51.9% vs 41.5%, *P* > 0.05). The prevalence of CMV infection in ANCA-positive BA patients was higher than that in the ANCA-negative BA patients (54.3% vs 37.8%, *P* > 0.05). There was no statistical relationship found between the presence of these autoantibodies and CMV infection.

Regardless of the severity of fibrosis, anti-M2-3E, ANA, or ANCA was present in the sera of BA patients. The positive rates of these autoantibodies showed no difference according to the extent of fibrosis (F0-F4) in the 124 BA patients (anti-M2-3E: 25.0%, 27.0%, 16.7%, 32.0%, and 22.2%, respectively, *P* > 0.05; ANA: 25.0%, 5.4%, 12.5%, 10.0%, and 22.2%, respectively, *P* > 0.05; ANCA: 50.0%, 30.0%, 41.7%, 32.0%, and 44.4%, respectively, *P* > 0.05).

Interestingly, as presented in Table 5, the prevalence of ANCA showed a positive correlation to the occurrence of postoperative cholangitis (*r* = 0.61, *P* < 0.05). Thus, the probability of ANCA negativity in postoperative cholangitis is very low. ANCA positivity did not correlate with the persistence of jaundice or acute liver injury, nor did anti-M2-3E positivity or ANA positivity show a statistical association with prognosis associated with the procedure.

The longitudinal (follow-up) findings for autoantibodies showed that 24 BA patients exhibited slight variation from the first evaluation to re-assessment within an average of 6 mo, but without statistically significant differences between the paired samples.

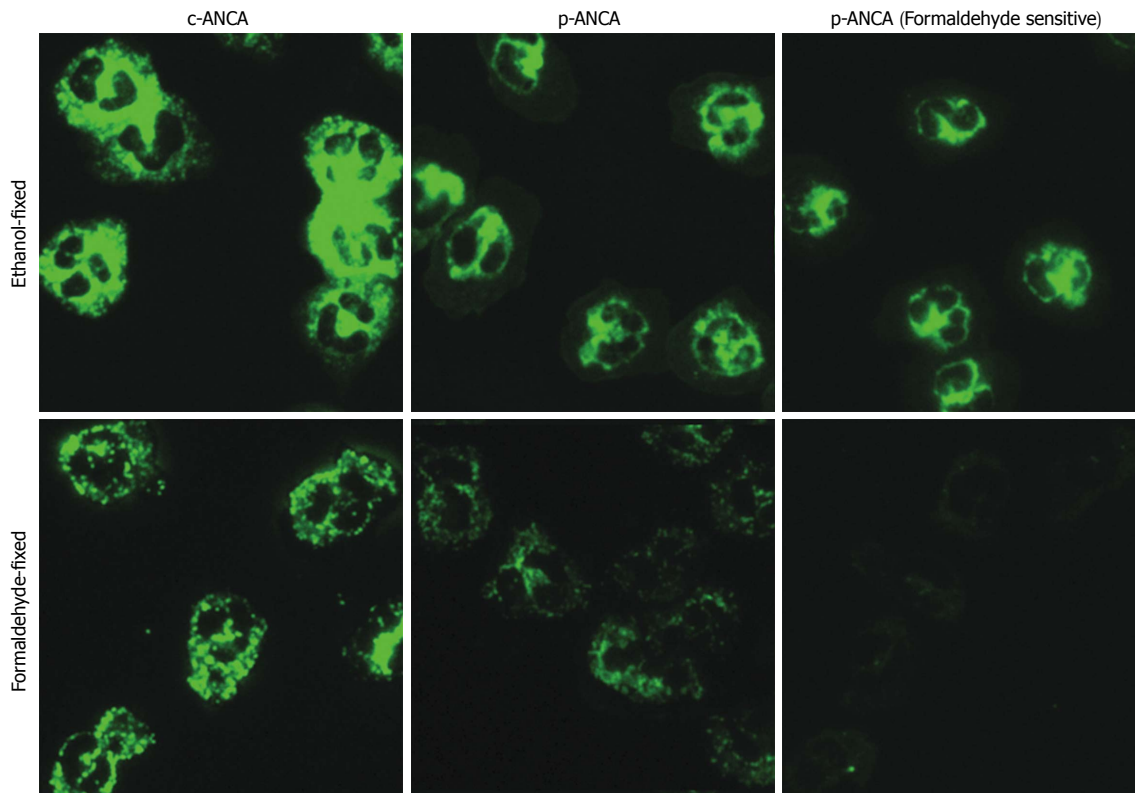
## DISCUSSION

In an earlier study, Hadchouel *et al*<sup>[11]</sup> found IgG deposits on the basement membranes of glandular formations

**Table 5 Association of autoantibodies and prognosis of Kasai procedure with follow-up of 52 biliary atresia patients *n* (%)**

Parameter	Anti-M2-3E		ANA		ANCA	
	Positive, <i>n</i> = 17	Negative, <i>n</i> = 35	Positive, <i>n</i> = 8	Negative, <i>n</i> = 44	Positive, <i>n</i> = 29	Negative, <i>n</i> = 23
TB, $\mu\text{mol/L}$	105.5 $\pm$ 80.3	109.4 $\pm$ 106.8	41.6 $\pm$ 49.5	127.9 $\pm$ 135.0	133.0 $\pm$ 120.7	85.3 $\pm$ 66.2
ALT, U/L	184.1 $\pm$ 119.8	171.7 $\pm$ 121.4	127.9 $\pm$ 135.0	185.8 $\pm$ 115.7	171.46 $\pm$ 95.5	179.6 $\pm$ 140.3
Occurrence of cholangitis	9 (52.9)	20 (57.1)	5 (62.5)	24 (54.5)	24 (82.8) <sup>a</sup>	5 (21.7) <sup>a</sup>

Data are described as mean  $\pm$  SD. <sup>a</sup>*P* < 0.05, *r* = 0.61; ANCA showed a positive correlation to the occurrence of postoperative cholangitis. TB is showed as a parameter of persistence of jaundice (TB > 34  $\mu\text{mol/L}$ ); ALT is showed as a parameter of acute liver injury (ALT > 35 U/L). ANCAs: Any positivity of ANCAs or specific ANCAs. ALT: Alanine aminotransferase; ANA: Anti-nuclear antibody; ANCA: Anti-neutrophil cytoplasmic antibody; BA: Biliary atresia; TB: Total bilirubin.



**Figure 3** The main fluorescence patterns of anti-neutrophil cytoplasmic antibodies in biliary atresia patients. ANCA detection by indirect immunofluorescence assays was performed on ethanol-fixed (upper panel) and formaldehyde-fixed (lower panel) human neutrophils. Depending on whether reactivities of formaldehyde-fixed human neutrophils were positive or not, p-ANCA was divided into p-ANCA with formaldehyde resistance and p-ANCA with formaldehyde sensitivity. ANCA: Anti-neutrophil cytoplasmic antibody; BA: Biliary atresia; (c)-ANCA: Cytoplasmic-ANCA; (p)-ANCA: Perinuclear-ANCA.

in about one-third of biliary remnants studied. Accumulating evidence suggests that the outset of BA may be triggered by an initial perinatal hepatobiliary viral infection<sup>[7,17]</sup>. Studies have also demonstrated that CMV infection initiates the autoimmune process in BA by targeting intrahepatic biliary epithelial cells of the host, and that the only reactivity to the  $\alpha$ -enolase antibody identified in BA was non-specific in human BA<sup>[10,18-20]</sup>. To date, serological specificity is too low for the diagnosis of BA, making it impossible to avoid damage from invasive surgery. Thus, the aim of this study was to seek a non-invasive biomarker which can discriminate BA from other causes of neonatal cholestasis or predict prognosis.

In the present study, 56.5% of BA patients were positive for at least one type of the autoantibodies.

The presence of autoantibodies in the BA patients was not an epiphenomenon, and high prevalence of autoantibodies in BA provided evidence of an autoimmune-mediated pathogenesis.

For PBC-related autoantibodies, 18.5% of the BA patients showed positivity for one or more autoantibodies. Particularly, anti-BPO was present in 14.8% (12/81) of the BA patients, which was detected at a higher frequency than that in the non-BA controls or healthy subjects but at a lower frequency than that in the PBC patients (84.5%)<sup>[21]</sup>. To our knowledge, this was the first time that anti-BPO has been detected in BA infants, as it is often reported in adults but has been rarely reported in pediatric patients<sup>[22]</sup>. Compared with PBC patients, the sensitivity of both AMA-M2 and

anti-BPO was relatively lower in BA patients, and the positivity of anti-BPO was higher than that of AMA-M2. This finding hinted that the corresponding antigen epitope of AMA in BA serum was different from that in PBC serum, whose reaction to E2 subunits of the oxo-glutarate dehydrogenase complex and branched-chain oxo-acid dehydrogenase complex was higher than that to PDC-E2. By enlarging the sample size and adopting ELISA to further confirm the detection of AMA in BA, we determined that 32 (25.8%) of 124 BA patients had a positive reaction for anti-M2-3E. Yet, the markedly increased anti-M2-3E positivity was not associated with CMV infection, severity of liver fibrosis, or prognosis of the Kasai procedure in BA.

It has been reported that anti-PML and anti-Sp100 are co-immunogenic in PBC patients, correlating with an unfavorable disease course and fast progression<sup>[23,24]</sup>. Our findings showed that the positivity for anti-Sp100, anti-gp210, and anti-PML in BA was rather low, and the co-existence of anti-Sp100 and anti-PML only occurred in a single BA patient, whose liver damage developed rapidly and seriously according to the diagnosis of pathological biopsy. Anti-gp210 is related to a severe disease course and poor prognosis of PBC. PBC patients who are positive for anti-gp210 will progress into liver failure more easily<sup>[25]</sup>. In our study, a total of four study participants displayed anti-gp210 positivity, including two with BA, one with choledochal cysts, and one healthy infant. The two patients with BA presented with biliary cirrhosis, while the one patient with choledochal cysts showed middle-stage liver damage. We were unsure whether the anti-gp210 in the healthy infant appeared earlier than the clinical manifestations, possibly as an alerting signal, similar to AMA-M2 in PBC. The AIH-related antibodies showed low positive rates in both BA and control groups. Among these autoantibodies, anti-LKM was not detected in any of the BA patients, which is consistent with the previous report by A-Kader *et al.*<sup>[26]</sup>.

For ANA, our study detected it at a higher frequency (11.3%) in BA patients than the previous report of 5%<sup>[26]</sup>. The prevalence was also significantly higher than that in patients with other liver diseases in our study. ANA, presenting mostly with a speckled pattern, is reportedly positive in approximately 6% of healthy children, which is similar to that found in the disease controls and healthy controls of our study<sup>[27,28]</sup>. As is known, ANA is fundamental for the diagnosis of a variety of autoimmune diseases, and the clinical value of ANA testing for autoimmune liver diseases is beyond doubt<sup>[29,30]</sup>. In our study, neither ANA nor specific ANAs exhibited any correlation to stages of liver fibrosis or prognosis of the KP procedure.

Both the positivity and titer of ANA are related to age of the autoimmune disease patient (being lower in childhood disease than in adulthood disease)<sup>[31,32]</sup>. Of note, the average age of BA patients in our study was 2.9 mo, a period in which nearly all of the IgG is maternally-derived. Thus, it is possible that maternally-

derived IgG may cause development of the disease. This is in line with the findings of Hannam *et al.*<sup>[33]</sup>, who documented cases where placental transfer of maternal AMA was associated with neonatal liver disease. Based on these data, longitudinal analysis of ANA in BA patients was performed (comparison of detection at the first evaluation and at re-assessment within an average 6 mo of follow-up). There were no significant differences between the preoperative and the postoperative findings, suggesting that the autoantibodies that arose in the BA patients may not be transient.

In addition, the prevalence of ANCA (29.0%) was remarkably higher in sera from BA patients than in that of healthy controls, similar to the positivity rates detected for the special ANCAs, which included anti-MPO and anti-PR3. However, the detection of ANCA in BA did not correspond fully with other reports. In a preliminary observation<sup>[34]</sup>, ANCA was detected in 91% of patients with BA, and was suggested as a promising biomarker for an immune-mediated process directed against specific hepatobiliary antigens; however, in another report by A-Kader *et al.*<sup>[26]</sup>, the ANCA was positive in only 1 of 20 patients. These differences in findings might reflect the differences in age of the patients studied or in the study methods applied.

In our study, we found that ANCA positivity in BA patients appeared more frequently with postoperative cholangitis than did ANCA negativity. As is known, ANCA is not only a helpful tool for establishing the diagnosis of Wegener's granulomatosis and microscopic polyangiitis but also appears with non-vascular chronic inflammatory diseases. The presence of ANCA has also been associated with the occurrence of relapses in AIH, with decreased liver synthesis function in PBC and with increased cholestasis in primary sclerosing cholangitis<sup>[12,35]</sup>. One of the minor antigen targets of ANCA present in BA has been shown to be  $\alpha$ -enolase, which is an enzyme involved in glycolysis and is ubiquitously expressed in a variety of cells, including biliary epithelial cells and hepatocytes<sup>[12]</sup>. It hinted that neutrophils may be activated in BA. The priming of neutrophils with tumor necrosis factor- $\alpha$  induces marked expression of ANCA antigens on the cell surface<sup>[36,37]</sup>. The expression of these target antigens facilitates the interaction with ANCA, resulting in subsequent polymorphonuclear leukocyte activation, which then induces production of reactive oxygen species, release of superoxide, and degranulation of neutrophils. ANCA was also reported to be involved in fibrosis of the lung and kidney<sup>[38,39]</sup>; however, we found that the rates of ANCA positivity were not significantly different in BA patients with fibrosis from stages F0 to F4, indicating that these antibodies might not be associated with the severity of liver fibrosis necessarily.

In summary, we found that the prevalence of ANCA increased prominently in BA, which showed a positive correlation to the occurrence of postoperative cholangitis. Although anti-M2-3E was not associated with prognosis



of the KP procedure, its high prevalence provided evidence of autoimmune activity in the pathogenesis of BA. Thus, autoantibodies in sera of BA children were not an accidental epiphenomenon. Future research in multi-centers focusing on identifying potentially pathogenic, bile duct-specific autoantibodies and the titer and source of autoantibodies in BA should be pursued.

## ARTICLE HIGHLIGHTS

### Research background

Accumulating evidence supports the biliary atresia (BA) pathogenesis theory of a primary perinatal viral trigger that is followed by an aberrant autoimmune-mediated attack on bile duct epithelia, resulting in inflammatory and fibrosing obstruction of the bile ducts. Similarly, both primary biliary cholangitis and autoimmune hepatitis are characterized by autoimmune-mediated injury to liver cells and biliary ducts, and autoimmune liver disease (ALD)-related autoantibodies play a crucial role in the accurate classification for such. The authors hypothesized that ALD-related autoantibodies would be also associated with BA.

### Research motivation

No previous study has comprehensively evaluated the prevalence of ALD-related autoantibodies and their roles in prognosis of BA. The authors attempted to explore the ALD-related autoantibodies in BA sera.

### Research objectives

The authors tried to search appropriate non-invasive biomarker for diagnosis and prognosis of BA.

### Research methods

The authors collected the sera of BA children and evaluated the prevalence of ALD-related autoantibodies using multiple methods, including the autoimmune liver diseases profile and specific anti-nuclear antibodies (ANAs) by line-blot assay; ANA and anti-neutrophil cytoplasmic antibody (ANCA) by indirect immunofluorescence assay; specific ANCAs and anti-M2-3E by enzyme linked immunosorbent assay. Simultaneously, associations of these autoantibodies with the clinical features of followed BA children were evaluated by Spearman's correlation coefficient.

### Research results

The overall positivity of serum ALD-related autoantibodies in BA patients was 56.5%. The prevalence of anti-M2-3E, ANA, and ANCA was significantly increased in a large cohort of infants with BA. In addition, the authors found that the ANCA showed a positive correlation to the occurrence of postoperative cholangitis.

### Research conclusions

High prevalence of ALD-related autoantibodies in the BA developmental process strongly reveals the autoimmune-mediated pathogenesis. ANCA positivity may be a useful serum biomarker to predict postoperative cholangitis of BA.

### Research perspectives

Our future research will focus on identifying potentially pathogenic, bile duct-specific autoantibodies and the titer and source of autoantibodies in BA.

## REFERENCES

- Hartley JL, Davenport M, Kelly DA. Biliary atresia. *Lancet* 2009; **374**: 1704-1713 [PMID: 19914515]
- Nizery L, Chardot C, Sissaoui S, Capito C, Henrion-Caude A, Debray D, Girard M. Biliary atresia: Clinical advances and perspectives. *Clin Res Hepatol Gastroenterol* 2016; **40**: 281-287 [PMID: 26775892 DOI: 10.1016/j.clinre.2015.11.010]
- Pakarinen MP, Rintala RJ. Surgery of biliary atresia. *Scand J Surg* 2011; **100**: 49-53 [PMID: 21482505 DOI: 10.1177/145749691110000109]
- Lykavieris P, Chardot C, Sokhn M, Gauthier F, Valayer J, Bernard O. Outcome in adulthood of biliary atresia: a study of 63 patients who survived for over 20 years with their native liver. *Hepatology* 2005; **41**: 366-371 [PMID: 15660386 DOI: 10.1002/hep.20547]
- Sokol RJ, Mack C, Narkewicz MR, Karrer FM. Pathogenesis and outcome of biliary atresia: current concepts. *J Pediatr Gastroenterol Nutr* 2003; **37**: 4-21 [PMID: 12827000 DOI: 10.1097/00005176-200307000-00003]
- Mack CL. What Causes Biliary Atresia? Unique Aspects of the Neonatal Immune System Provide Clues to Disease Pathogenesis. *Cell Mol Gastroenterol Hepatol* 2015; **1**: 267-274 [PMID: 26090510 DOI: 10.1016/j.jcmgh.2015.04.001]
- Harada K. Sclerosing and obstructive cholangiopathy in biliary atresia: mechanisms and association with biliary innate immunity. *Pediatr Surg Int* 2017; **33**: 1243-1248 [PMID: 29039048 DOI: 10.1007/s00383-017-4154-8]
- Mohanty SK, Donnelly B, Lobeck I, Walther A, Dupree P, Coots A, Meller J, McNeal M, Sestak K, Tiao G. The SRL peptide of rhesus rotavirus VP4 protein governs cholangiocyte infection and the murine model of biliary atresia. *Hepatology* 2017; **65**: 1278-1292 [PMID: 27859498 DOI: 10.1002/hep.28947]
- Shimada T, Imaizumi T, Shirai K, Tatsuta T, Kimura T, Hayakari R, Yoshida H, Matsumiya T, Kijima H, Mizukami H, Hakamada K. CCL5 is induced by TLR 3 signaling in HuCCT1 human biliary epithelial cells: possible involvement in the pathogenesis of biliary atresia. *Biomed Res* 2017; **38**: 269-276 [PMID: 29070776 DOI: 10.2220/biomedres.38.269]
- Xu Y, Yu J, Zhang R, Yin Y, Ye J, Tan L, Xia H. The perinatal infection of cytomegalovirus is an important etiology for biliary atresia in China. *Clin Pediatr (Phila)* 2012; **51**: 109-113 [PMID: 22144720 DOI: 10.1177/0009922811406264]
- Hadchouel M, Hugon RN, Odievre M. Immunoglobulin deposits in the biliary remnants of extrahepatic biliary atresia: a study by immunoperoxidase staining in 128 infants. *Histopathology* 1981; **5**: 217-221 [PMID: 7216182 DOI: 10.1111/j.1365-2559.1981.tb01779.x]
- Lu BR, Brindley SM, Tucker RM, Lambert CL, Mack CL.  $\alpha$ -enolase autoantibodies cross-reactive to viral proteins in a mouse model of biliary atresia. *Gastroenterology* 2010; **139**: 1753-1761 [PMID: 20659472 DOI: 10.1053/j.gastro.2010.07.042]
- Peng X, Yang L, Liu H, Pang S, Chen Y, Fu J, Chen Y, Wen Z, Zhang R, Zhu B, Yu J, Invernizzi P. Identification of Circulating MicroRNAs in Biliary Atresia by Next-Generation Sequencing. *J Pediatr Gastroenterol Nutr* 2016; **63**: 518-523 [PMID: 26960174 DOI: 10.1097/MPG.0000000000001194]
- Sarin SK, Kedarisetty CK, Abbas Z, Amarapurkar D, Bihari C, Chan AC, Chawla YK, Dokmeci AK, Garg H, Ghazinyan H, Hamid S, Kim DJ, Komolmit P, Lata S, Lee GH, Lesmana LA, Mahtab M, Maiwall R, Moreau R, Ning Q, Pamecha V, Payawal DA, Rastogi A, Rahman S, Rela M, Saraya A, Samuel D, Saraswat V, Shah S, Shiha G, Sharma BC, Sharma MK, Sharma K, Butt AS, Tan SS, Vashishtha C, Wani ZA, Yuen MF, Yokosuka O; APASL ACLF Working Party. Acute-on-chronic liver failure: consensus recommendations of the Asian Pacific Association for the Study of the Liver (APASL) 2014. *Hepatol Int* 2014; **8**: 453-471 [PMID: 26202751 DOI: 10.1007/s12072-014-9580-2]
- Huang Y, Wang Z, Liao B, Liang JY, Zhou LY, Wang F, Li W, Liu JY, Xie XY, Lu MD, Liu GJ, Wang W. Assessment of liver fibrosis in chronic hepatitis B using acoustic structure quantification: quantitative morphological ultrasound. *Eur Radiol* 2016; **26**: 2344-2351 [PMID: 26486937 DOI: 10.1007/s00330-015-4056-x]
- Yang LY, Fu J, Peng XF, Pang SY, Gao KK, Chen ZR, He LJ, Wen Z, Wang H, Li L, Wang FH, Yu JK, Xu Y, Gong ST, Xia HM, Liu HY. Validation of aspartate aminotransferase to platelet ratio for diagnosis of liver fibrosis and prediction of postoperative prognosis in infants with biliary atresia. *World J Gastroenterol* 2015; **21**: 5893-5900 [PMID: 26019453 DOI: 10.3748/wjg.v21.i19.5893]

- 17 **Mack CL.** The pathogenesis of biliary atresia: evidence for a virus-induced autoimmune disease. *Semin Liver Dis* 2007; **27**: 233-242 [PMID: 17682970 DOI: 10.1055/s-2007-985068]
- 18 **Wen J,** Xiao Y, Wang J, Pan W, Zhou Y, Zhang X, Guan W, Chen Y, Zhou K, Wang Y, Shi B, Zhou X, Yuan Z, Cai W. Low doses of CMV induce autoimmune-mediated and inflammatory responses in bile duct epithelia of regulatory T cell-depleted neonatal mice. *Lab Invest* 2015; **95**: 180-192 [PMID: 25531565 DOI: 10.1038/labinvest.2014.148]
- 19 **Terrier B,** Degand N, Guilpain P, Servettaz A, Guillemin L, Mouthon L. Alpha-enolase: a target of antibodies in infectious and autoimmune diseases. *Autoimmun Rev* 2007; **6**: 176-182 [PMID: 17289554 DOI: 10.1016/j.autrev.2006.10.004]
- 20 **Zani A,** Quaglia A, Hadzić N, Zuckerman M, Davenport M. Cytomegalovirus-associated biliary atresia: An aetiological and prognostic subgroup. *J Pediatr Surg* 2015; **50**: 1739-1745 [PMID: 25824438 DOI: 10.1016/j.jpedsurg.2015.03.001]
- 21 **Villalta D,** Sorrentino MC, Girolami E, Tampoia M, Alessio MG, Brusca I, Daves M, Porcelli B, Barberio G, Bizzaro N; Study Group on Autoimmune Diseases of the Italian Society of Laboratory Medicine. Autoantibody profiling of patients with primary biliary cirrhosis using a multiplexed line-blot assay. *Clin Chim Acta* 2015; **438**: 135-138 [PMID: 25172039 DOI: 10.1016/j.cca.2014.08.024]
- 22 **Gregorio GV,** Portmann B, Mowat AP, Vergani D, Mieli-Vergani G. A 12-year-old girl with antimitochondrial antibody-positive autoimmune hepatitis. *J Hepatol* 1997; **27**: 751-754 [PMID: 9365052 DOI: 10.1016/S0168-8278(97)80093-1]
- 23 **Sternsdorf T,** Guldner HH, Szostek C, Grötzing T, Will H. Two nuclear dot-associated proteins, PML and Sp100, are often co-autoimmunogenic in patients with primary biliary cirrhosis. *Scand J Immunol* 1995; **42**: 257-268 [PMID: 7631159 DOI: 10.1111/j.1365-3083.1995.tb03652.x]
- 24 **Muratori P,** Muratori L, Ferrari R, Cassani F, Bianchi G, Lenzi M, Rodrigo L, Linares A, Fuentes D, Bianchi FB. Characterization and clinical impact of antinuclear antibodies in primary biliary cirrhosis. *Am J Gastroenterol* 2003; **98**: 431-437 [PMID: 12591064 DOI: 10.1111/j.1572-0241.2003.07257.x]
- 25 **Gao L,** Tian X, Liu B, Zhang F. The value of antinuclear antibodies in primary biliary cirrhosis. *Clin Exp Med* 2008; **8**: 9-15 [PMID: 18385935 DOI: 10.1007/s10238-008-0150-6]
- 26 **A-Kader HH,** Abdel-Hameed A, Al-Shabrawi M, Mohsen N, El-Karakasy H, Hassanein B, Elsayed B, Abdel-Khalik MK, Karjoo M. Is biliary atresia an autoimmune disease? *Eur J Gastroenterol Hepatol* 2003; **15**: 447 [PMID: 12655270 DOI: 10.1097/01.meg.0000050021.68425.6c]
- 27 **Wananukul S,** Voramethkul W, Kaewopas Y, Hanvivatvong O. Prevalence of positive antinuclear antibodies in healthy children. *Asian Pac J Allergy Immunol* 2005; **23**: 153-157 [PMID: 16252846]
- 28 **Hilário MO,** Len CA, Roja SC, Terreri MT, Almeida G, Andrade LE. Frequency of antinuclear antibodies in healthy children and adolescents. *Clin Pediatr (Phila)* 2004; **43**: 637-642 [PMID: 15378151 DOI: 10.1177/000992280404300709]
- 29 **Agmon-Levin N,** Damoiseaux J, Kallenberg C, Sack U, Witte T, Herold M, Bossuyt X, Musset L, Cervera R, Plaza-Lopez A, Dias C, Sousa MJ, Radice A, Eriksson C, Hultgren O, Viander M, Khamashta M, Regenass S, Andrade LE, Wiik A, Tincani A, Rönnelid J, Bloch DB, Fritzler MJ, Chan EK, Garcia-De La Torre I, Konstantinov KN, Lahita R, Wilson M, Vainio O, Fabien N, Sinico RA, Meroni P, Shoenfeld Y. International recommendations for the assessment of autoantibodies to cellular antigens referred to as anti-nuclear antibodies. *Ann Rheum Dis* 2014; **73**: 17-23 [PMID: 24126457 DOI: 10.1136/annrheumdis-2013-203863]
- 30 **Damoiseaux J,** von Mühlen CA, Garcia-De La Torre I, Carballo OG, de Melo Cruvinel W, Francescantonio PL, Fritzler MJ, Herold M, Mimori T, Satoh M, Andrade LE, Chan EK, Conrad K. International consensus on ANA patterns (ICAP): the bumpy road towards a consensus on reporting ANA results. *Auto Immun Highlights* 2016; **7**: 1 [PMID: 26831867 DOI: 10.1007/s13317-016-0075-0]
- 31 **Rosenberg JN,** Johnson GD, Holborow EJ, Bywaters EG. Eosinophil-specific and other granulocyte-specific antinuclear antibodies in juvenile chronic polyarthritis and adult rheumatoid arthritis. *Ann Rheum Dis* 1975; **34**: 350-353 [PMID: 1081376 DOI: 10.1136/ard.34.4.350]
- 32 **PrabhuDas M,** Adkins B, Gans H, King C, Levy O, Ramilo O, Siegrist CA. Challenges in infant immunity: implications for responses to infection and vaccines. *Nat Immunol* 2011; **12**: 189-194 [PMID: 21321588 DOI: 10.1038/ni0311-189]
- 33 **Hannam S,** Bogdanos DP, Davies ET, Hussain MJ, Portmann BC, Mieli-Vergani G, Vergani D. Neonatal liver disease associated with placental transfer of anti-mitochondrial antibodies. *Autoimmunity* 2002; **35**: 545-550 [PMID: 12765481 DOI: 10.1080/089169302100054057]
- 34 **Vasiliauskas EA,** Cobb L, Vidrich A, Targan SR, Rosenthal P. Biliary atresia - an autoimmune disorder? *Journal of Pediatric Gastroenterology and Nutrition* 1995; **21**: 327 [DOI: 10.1097/00005176-199510000-00025]
- 35 **Roosendaal C,** de Jong MA, van den Berg AP, van Wijk RT, Limburg PC, Kallenberg CG. Clinical significance of anti-neutrophil cytoplasmic antibodies (ANCA) in autoimmune liver diseases. *J Hepatol* 2000; **32**: 734-741 [PMID: 10845659 DOI: 10.1016/S0168-8278(00)80241-X]
- 36 **Ewert BH,** Jennette JC, Falk RJ. Anti-myeloperoxidase antibodies stimulate neutrophils to damage human endothelial cells. *Kidney Int* 1992; **41**: 375-383 [PMID: 1313124 DOI: 10.1038/ki.1992.52]
- 37 **Falk RJ,** Terrell RS, Charles LA, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. *Proc Natl Acad Sci USA* 1990; **87**: 4115-4119 [PMID: 2161532 DOI: 10.1073/pnas.87.11.4115]
- 38 **Hosoda C,** Baba T, Hagiwara E, Ito H, Matsuo N, Kitamura H, Iwasawa T, Okudela K, Takemura T, Ogura T. Clinical features of usual interstitial pneumonia with anti-neutrophil cytoplasmic antibody in comparison with idiopathic pulmonary fibrosis. *Respirology* 2016; **21**: 920-926 [PMID: 26994375 DOI: 10.1111/resp.12763]
- 39 **Herivier B,** Pagnoux C, Agard C, Haroche J, Amoura Z, Guillevin L, Hamidou MA; French Vasculitis Study Group. Pulmonary fibrosis associated with ANCA-positive vasculitides. Retrospective study of 12 cases and review of the literature. *Ann Rheum Dis* 2009; **68**: 404-407 [PMID: 18957485 DOI: 10.1136/ard.2008.096131]

**P- Reviewer:** Govindarajan GK, Watanabe T **S- Editor:** Chen K  
**L- Editor:** Wang TQ **E- Editor:** Li D



## Observational Study

# *Helicobacter pylori* and corpus gastric pathology are associated with lower serum ghrelin

Paula Mantero, Gonzalo Sebastián Matus, Rodolfo Ernesto Corti, Ana María Cabanne, Gerardo Gabriel Zerbetto de Palma, Liliana Marchesi Olid, María Marta Piskorz, Marcela Beatriz Zubillaga, Mariana Andrea Janjetic, Cinthia Gabriela Goldman

Paula Mantero, Gerardo Gabriel Zerbetto de Palma, Marcela Beatriz Zubillaga, Mariana Andrea Janjetic, Cinthia Gabriela Goldman, Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Física, Buenos Aires C1113AAD, Argentina

Gonzalo Sebastián Matus, Rodolfo Ernesto Corti, Hospital de Gastroenterología "Dr. Carlos Bonorino Udaondo", Sección Esófago-Estómago, Buenos Aires C1264AAA, Argentina

Ana María Cabanne, Hospital de Gastroenterología "Dr. Carlos Bonorino Udaondo", Unidad Patología, Buenos Aires C1264AAA, Argentina

Gerardo Gabriel Zerbetto de Palma, Universidad de Buenos Aires - CONICET, Facultad de Medicina, Instituto de Microbiología y Parasitología Médica (IMPAM), Buenos Aires C1121ABG, Argentina

Liliana Marchesi Olid, Mariana Andrea Janjetic, Universidad de Buenos Aires, Facultad de Medicina, Escuela de Nutrición, Buenos Aires C1121ABG, Argentina

María Marta Piskorz, Hospital de Clínicas "José de San Martín", División Gastroenterología, Buenos Aires C1120AAR, Argentina

Marcela Beatriz Zubillaga, Mariana Andrea Janjetic, Cinthia Gabriela Goldman, National Scientific and Technical Research Council (CONICET), Buenos Aires C1425FQB, Argentina

ORCID number: Paula Mantero (0000-0003-3509-1195); Gonzalo Sebastián Matus (0000-0001-6624-2705); Rodolfo Ernesto Corti (0000-0002-6573-3075); Ana María Cabanne (0000-0003-3567-8180); Gerardo Gabriel Zerbetto de Palma (0000-0003-4235-8978); Liliana Marchesi Olid (0000-0001-8277-8972); María Marta Piskorz (0000-0001-8616-3457); Marcela Beatriz Zubillaga (0000-0002-7331-6722); Mariana Andrea Janjetic (0000-0001-5496-3951); Cinthia Gabriela Goldman

(0000-0001-7287-1372).

**Author contributions:** Janjetic MA and Goldman CG formulated the research questions and designed the study; Mantero P, Matus GS, Corti RE, Zerbetto de Palma GG, Marchesi Olid L and Piskorz MM collected samples and data; Mantero P, Cabanne AM, Zerbetto de Palma GG and Goldman CG performed experiments; Janjetic MA performed the statistical analysis; Mantero P, Zubillaga MB, Janjetic MA and Goldman CG interpreted data; Mantero P, Janjetic MA and Goldman CG wrote the article; all authors have read and approved the final manuscript.

**Supported by the Universidad de Buenos Aires (UBA),** Buenos Aires, Argentina, No. UBACYT 20020100100837 and No. UBACYT 20020130100645BA to Goldman CG; and the International Atomic Energy Agency (IAEA), Vienna, Austria, Coordinated Research Project (CRP) E43025 No. ARG-16746 to Goldman CG. UBA and IAEA had no role in the design, analysis or writing of the present article.

**Institutional review board statement:** The study was reviewed and approved by the Hospital de Gastroenterología "Dr. Carlos Bonorino Udaondo" and the Hospital de Clínicas "José de San Martín" Institutional Review Boards.

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

**Data sharing statement:** No additional data are available.

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Manuscript source: Unsolicited manuscript

Correspondence to: Cinthia Gabriela Goldman, PhD, Adjunct Professor, Research Scientist, Physics Department, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, Buenos Aires C1113AAD, Argentina. [cgold@ffyb.uba.ar](mailto:cgold@ffyb.uba.ar)  
Telephone: +54-11-52874551  
Fax: +54-11-52874563

Received: November 4, 2017

Peer-review started: November 4, 2017

First decision: November 14, 2017

Revised: November 30, 2017

Accepted: December 4, 2017

Article in press: December 4, 2017

Published online: January 21, 2018

## Abstract

### AIM

To evaluate the association of *Helicobacter pylori* (*H. pylori*), *cagA* genotype, and type of gastric pathology with ghrelin, leptin and nutritional status.

### METHODS

Fasted dyspeptic adults (18-70 years) referred for an upper digestive endoscopy were enrolled in this cross-sectional study. Height and weight were assessed for body mass index (BMI) calculation. A sociodemographic survey was administered and nutrient intake was evaluated with 24 h dietary recalls. Serum total ghrelin and leptin levels were analyzed by enzyme-linked immunosorbent assay. <sup>13</sup>C-Urea Breath Test was performed and four gastric biopsies were obtained during endoscopy for histopathology and *H. pylori* DNA amplification and genotyping. Data analysis was performed using  $\chi^2$ , Mann-Whitney *U*, Kruskal-Wallis tests, Spearman's correlation and linear regression.

### RESULTS

One hundred and sixty-three patients (40.8 ± 14.0 years), 98/65 females/males, were included. Overall, persistent *H. pylori* prevalence was 53.4% (95%CI: 45.7%-65.8%). Neither nutrient intake nor BMI differed significantly between *H. pylori* positive and negative groups. Serum ghrelin was significantly lower in infected patients [median 311.0 pg/mL (IQR 230.0-385.5)] than in uninfected ones [median 355.0 pg/mL (IQR 253.8-547.8)] (*P* = 0.025), even after adjusting for BMI and gender (*P* = 0.03). Ghrelin levels tended to be lower in patients carrying *cagA* positive strains both in the antrum and the corpus; however, differences with those carrying *cagA* negative strains did not reach statistical significance (*P* = 0.50 and *P* = 0.49, respectively). In addition, the type and severity of gastric pathology in the corpus was associated with lower serum ghrelin (*P* = 0.04), independently of *H.*

*pylori* status. Conversely, leptin levels did not differ significantly between infected and uninfected patients [median 1.84 ng/mL (0.80-4.85) *vs* 1.84 ng/mL (0.50-5.09), (*P* = 0.51)].

### CONCLUSION

*H. pylori* infection and severity of gastric corpus pathology are associated with lower serum ghrelin. Further studies could confirm a lower ghrelin prevalence in *cagA*-positive patients.

**Key words:** *Helicobacter pylori*; *cagA*; Ghrelin; Leptin; Pathology

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**Core tip:** The relationship between *Helicobacter pylori* (*H. pylori*) infection and hormonal modulation of food intake is still controversial. We conducted this study to evaluate the association between *H. pylori* infection, the genotype of infecting strains and the type of gastric pathology, with serum ghrelin and leptin concentrations and anthropometric nutritional status of dyspeptic patients. Our study demonstrated that *H. pylori* infection and the severity of gastric pathology of the corpus are associated with lower ghrelin serum concentrations. We also observed lower, but not significantly different, ghrelin levels in patients carrying *cagA* positive strains, an observation that should be evaluated further in future studies.

Mantero P, Matus GS, Corti RE, Cabanne AM, Zerbetto de Palma GG, Marchesi Olid L, Piskorz MM, Zubillaga MB, Janjetic MA, Goldman CG. *Helicobacter pylori* and corpus gastric pathology are associated with lower serum ghrelin. *World J Gastroenterol* 2018; 24(3): 397-407 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i3/397.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i3.397>

## INTRODUCTION

Since its discovery by Drs. Marshall and Warren<sup>[1]</sup>, the bacterium *Helicobacter pylori* (*H. pylori*) has been associated with the development of diverse gastroduodenal pathologies of the host, from chronic superficial gastritis to gastric cancer, being classified as a class I carcinogen for its definite role in this latter outcome<sup>[2,3]</sup>. It is already known that the outcome of *H. pylori* colonization is determined by the confluence of different factors, related not only with its presence within the stomach, but with colonizing strains, environmental factors, and susceptibility of the host<sup>[4,5]</sup>. Consequently, only a low proportion of *H. pylori* persistently infected individuals develop gastroduodenal pathology. Recent data demonstrate 85%-90% of *H. pylori* positive individuals are asymptomatic; 6%-20%



are at risk of developing peptic-ulcer disease; and 0.1%-1% develop gastric cancer<sup>[6]</sup>.

The stomach plays an important role in food intake regulation through the production of ghrelin and leptin, two neuroendocrine hormones which exert hypothalamic actions regulating appetite and satiety<sup>[7-9]</sup>. Ghrelin is an orexigenic hormone predominantly produced by the ghrelin producing cells in the gastric oxyntic mucosa, which stimulates food intake, decreases energy expenditure and promotes body weight gain<sup>[10]</sup>. Its main action at the central nervous system consists in the stimulation of growth hormone (GH) release, affecting several physiological processes. In the gastrointestinal tract, it stimulates gastrin release, gastric acid secretion and gastric emptying *via* vagal activation<sup>[11]</sup>. In contrast, leptin exerts anorexigenic effects by acting on the hypothalamus, suppressing food intake and increasing energy metabolism. Circulating leptin is mainly provided by adipocytes production; however, a low proportion is produced by chief and endocrine P cells in the gastric tissue<sup>[7]</sup>. Gastric leptin also regulates intestinal nutrient absorption, delays gastric emptying and signals short-term satiety<sup>[9]</sup>.

Recently, controversial results on the influence of *H. pylori* colonization on the gastric regulation of food intake and body mass index (BMI) have been reported. Some authors described lower gastric and/or plasmatic ghrelin levels in *H. pylori* positive patients<sup>[12-14]</sup>, whereas others reported similar ghrelin serum concentrations for *H. pylori* positive and negative individuals<sup>[15]</sup>, or even higher ghrelin gastric production in infected patients<sup>[16]</sup>. On the other hand, it has been described that leptin plasmatic concentrations are not effected by *H. pylori* colonization<sup>[14]</sup>; however, lower serum leptin levels have been reported in infected patients without a variation in gastric biopsies<sup>[15]</sup>. After a systematic review of the literature with a meta-analysis, Nweneka and Prentice concluded that circulating ghrelin levels were lower in *H. pylori* infected than in *H. pylori* negative individuals; nevertheless, its variation after *H. pylori* eradication remained controversial<sup>[17]</sup>. Two important aspects that would be involved in appetite hormone levels are the severity and topology of gastric mucosal affection, although these are not often evaluated. Liew *et al.*<sup>[12]</sup> and Chuang *et al.*<sup>[14]</sup> reported an association between chronic gastric inflammation histology scores and lower plasma ghrelin levels, and more recently an association has been described between plasma ghrelin levels and the severity of atrophy related to *H. pylori* infection in hemodialysis patients<sup>[18]</sup>. Evaluation of *H. pylori cagA* genotype would be also central because CagA protein producing strains have a higher interaction with the host<sup>[5]</sup>, exerting cellular effects including the induction of pro-inflammatory signals<sup>[19]</sup>; however, the association of *H. pylori* colonizing strains genotype with ghrelin levels has been scarcely studied<sup>[15,20]</sup>.

This present study was conducted to evaluate the association of persistent *H. pylori* infection, the genotype of infecting *H. pylori* strain, the type of gastric pathology and serum ghrelin and leptin concentrations, in dyspeptic patients with a known nutritional status.

## MATERIALS AND METHODS

### *Patients and ethics*

This Cross-Sectional study included 12-h fasted dyspeptic adults (18-70 years) referred for an upper digestive endoscopy to the Esophagus-Stomach Section of the Hospital de Gastroenterología "Dr. Carlos Bonorino Udaondo", and the Gastroenterology Unit of the Hospital de Clínicas "José de San Martín", both located in Buenos Aires City, Argentina. Inclusion criteria were the presence of upper gastrointestinal signs and symptoms (gastroesophageal reflux, oesophagitis, dyspepsia and abdominal pain), while exclusion criteria were antecedents of gastric surgery, neoplastic disease, diabetes, celiac disease, thyroid, renal or hepatic pathologies, drug abuse, coagulopathies, pregnancy, previous *H. pylori* treatment and use of antimicrobials or acid suppressants during the month before enrollment. The protocol was approved by the ethics committees of the two hospitals in which patients were recruited and it was conducted according to the guidelines laid down in the Declaration of Helsinki and the Guidelines of Good Clinical Practice. Written informed consent was obtained from the patients for inclusion in the study, in which the objectives, procedures and outcomes were detailed. Patients were informed about the results of the diagnostic tests and received the appropriate treatment after an individual basis.

### *Epidemiological questionnaire*

A sociodemographic survey was administered to the patients in order to obtain information about possible predictive variables for *H. pylori* positivity. The questionnaire was focused on ethnicity, socio-demographic factors and sanitary conditions.

### *Anthropometric indicators*

Body weight and height were obtained at enrollment to calculate the Body Mass Index (BMI) of each patient as their weight in kilograms divided by the square of their height in meters. Height was recorded using a stadiometer (Stanley, Morangis, France) to the nearest 0.1 cm, and weight was measured with a portable mechanical scale (CAM, Buenos Aires, Argentina) to the nearest 100 g. Underweight, stunting, overweight and obesity were defined according to the classification of the World Health Organization<sup>[21]</sup>. Anthropometric techniques were previously standardized according to the CDC anthropometry procedures manual<sup>[22]</sup>. Waist

circumference was measured with a stretch-resistant tape to determine abdominal adiposity as a predictor of cardiovascular disease risk<sup>[23]</sup>.

### Dietary assessment

Energy and macronutrient intake were assessed with 24 h dietary recalls administered to the patients. A book of picture charts was used to aid respondents in portion size estimation<sup>[24]</sup>. Data analysis was performed using the food composition database compiled in 2007 by the Argentine Ministry of Health<sup>[25]</sup>.

### <sup>13</sup>C-Urea Breath Test (<sup>13</sup>C-UBT)

The <sup>13</sup>C-UBT was performed using a commercial kit (TAU-KIT, Isomed Pharma, Madrid, Spain). Briefly, fasted patients were instructed to drink a 100 mL beverage enriched in citric acid. After 10 min, two pre-dose basal exhaled air samples were obtained in hermetically sealed containers. Each patient was given a 50 mL water solution in which a soluble tablet of 100 mg <sup>13</sup>C-urea had been dissolved. Two breath samples were collected after 30 min. Samples were measured in an isotope ratio mass spectrometer coupled to a gas chromatograph (Finnigan MAT GmbH, Thermo Fisher Scientific, Bremen, Germany) as previously described<sup>[26,27]</sup>. A change of 3.5‰ in the Delta Over Baseline values was considered positive<sup>[28]</sup>. Urea Hydrolysis Rate (UHR) was calculated from the <sup>13</sup>C-UBT to normalize the results by the endogenous CO<sub>2</sub> production per body size<sup>[29]</sup>, as previously described<sup>[30]</sup>.

### Ghrelin and leptin determination

Venous blood samples were collected just before endoscopy. Serum was obtained by centrifugation and kept at -80 °C until assay. Serum total ghrelin levels were analyzed in duplicate samples by Enzyme-linked Immunosorbent Assay (ELISA) using a commercial kit (EMD Millipore Corporation, MO, United States), and serum leptin concentrations with a commercial Enzyme Amplified Sensitivity Immunoassay (EASIA) kit (DIAsource ImmunoAssays SA, Belgium). Absorbance was measured in a plate reader (Multiskan EX, Thermo Scientific INC, United States) and the results were processed with the Cembal 2.2® program (Cembal Applications 2000-2001, Argentina) to calculate hormonal concentrations.

### Endoscopy

Subjects underwent a routine endoscopic evaluation of the upper gastrointestinal tract during which two gastric biopsies from the antrum and two from the corpus were obtained. One of the samples of each gastric site was used for histological assessment and the other one for molecular biology evaluation.

### Histological analysis

Gastric biopsies were processed with a spin tissue

processor (MicromSTP120, ThermoScientific Corp., Walldorf, Germany) comprising the following steps: Formol immersion (2 h), dehydration in alcohol 96% (6 h), alcohol 100% (4 h) and xylene (3 h), and paraffin immersion at 56-58 °C (3 h) and at 62 °C (3 h). Samples were then embedded in paraffin at 62 °C, from which 4 µm consecutive sections were obtained for haematoxylin-eosin and Giemsa histologic staining. Microscopic assessment was classified according to the updated Sydney System Classification<sup>[31]</sup>.

### PCR amplification and determination of *H. pylori* genotype

DNA was extracted from gastric biopsies from the antrum and corpus using the QIAamp Mini Kit (QIAGEN, INC., CA, United States) and evaluation of *H. pylori vacA* and *cagA* genotypes was performed by PCR amplification. Primer sequences (5'-3') and product base pair sizes were as follows: va1F (ATGGAAATACAACAAACACAC) and va1XR (CCTGAGACCGTTCCTACAGC) for *vacA*S1 allele (176 bp product) and *vacA*S2 (203 bp product)<sup>[32]</sup>; cagA22 (GATCCTGCTAGTTTGTCAGCGA) and cagA23 (CTTATCATTCACGAGTTTGAGC) for the *cagA* gene (127 bp product)<sup>[33]</sup>. Amplification was carried out in a total volume of 50 µL containing 1XTaq polymerase buffer, 1.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L (each) deoxynucleotide, 1.0 U of Platinum® Taq DNA Polymerase (Invitrogen Argentina, Buenos Aires, Argentina), 0.1 µg each oligonucleotide primer, and 5 µL of DNA template. PCR [94 °C for 3 min; 35 cycles of 94 °C for 30 s, 50 °C for 45 s (*vacA*) or 54 °C for 30 s (*cagA*), and 72 °C for 45 s (*vacA*) or for 30 s (*cagA*); 72 °C for 5 min] was performed with an automatic thermocycler (MyCycler, BioRad, CA, United States) and a 10 µL aliquot was analyzed by electrophoresis through a 1.5% (wt/vol) agarose gel stained with ethidium bromide. PCR products were visualized by excitation under UV light.

### *H. pylori* status determination

*H. pylori* infection was determined by the three methodologies described above: <sup>13</sup>C-UBT, histology and *vacA* PCR amplification from gastric biopsies. Patients were considered *H. pylori* positive with positive results from at least two of the three diagnostic methods.

### Statistical analysis

A sample size of 160 individuals was calculated to be included in the study using the StatCalc program (Epi Info Version 3.2, Georgia, United States), setting an  $\alpha$  error of 0.05, a  $\beta$  error of 0.20, an estimated 50% *H. pylori* infection prevalence in adult patients and a 25% expected frequency of ghrelin hormonal variation between the *H. pylori* positive and negative groups. Statistical analyses were performed by the  $\chi^2$ , Mann-Whitney *U* and Kruskal-Wallis tests, Spearman correlation and linear regression. Significance levels were set at  $\alpha < 0.05$ . Statistical analyses were performed

**Table 1** Nutrient intake according to *Helicobacter pylori* infection

Nutrient	<i>Helicobacter pylori</i> negative		<i>Helicobacter pylori</i> positive		P value
	Median	IQR	Median	IQR	
Energy (kcal/d)	1627.6	1187.4-2063.0	1748.4	1089.4-2308.8	0.65
Carbohydrate (g/d)	196.4	151.9-251.1	202.5	135.9-311.7	0.42
Protein (g/d)	72.7	47.6-88.5	68.8	42.7-104.6	0.91
Fat (g/d)	59.8	37.8-78.5	60.0	34.0-81.1	0.94

IQR: Interquartile range.

**Table 2** Anthropometric measurements and hormonal serum concentrations according to *Helicobacter pylori* status

Variable	<i>Helicobacter pylori</i> negative	<i>Helicobacter pylori</i> positive	P value
Weight (kg) <sup>1</sup>	66.43 (14.15)	69.52 (12.71)	0.08
Height (m) <sup>1</sup>	1.61 (0.09)	1.61 (0.08)	0.93
BMI (kg/m <sup>2</sup> ) <sup>1</sup>	25.78 (5.13)	26.93 (4.26)	0.09
Waist Circumference (cm) <sup>1</sup>	83.78 (11.89)	87.04 (11.09)	0.08
Ghrelin (pg/mL) <sup>2</sup>	355.0 (253.8-547.8)	311.0 (230.0-385.5)	0.025 <sup>a</sup>
Leptin (ng/mL) <sup>2</sup>	1.84 (0.50-5.09)	1.84 (0.80-4.85)	0.87

<sup>1</sup>mean (SD); <sup>2</sup>median (IQR); <sup>a</sup>P < 0.05, statistically significant (Mann-Whitney test).

med using SPSS software version 17.0 (IBM SPSS). The statistical methods of this study were reviewed by Janjetic MA from the Universidad de Buenos Aires and CONICET.

## RESULTS

### Epidemiology

The present study included 163 patients (40.8 ± 14.0 years of age), 98/65 females/males. Prevalence of *H. pylori* infection was 53.4% (95%CI: 45.7%-65.8%). *H. pylori* positive and negative patients did not differ significantly in terms of age (*P* = 0.48) or gender (*P* = 0.46). Sociodemographic variables which proved to be associated with the infection were ethnicity (*P* = 0.007), with a higher *H. pylori* prevalence in South American Indians, and poorer sanitary conditions denoted by the type of house (*P* = 0.01) and type of flooring (*P* = 0.03).

### Food intake

Dietary recalls were collected from all of the participating adults. Table 1 summarizes the macronutrient and energy intake of the patients according to *H. pylori* status. Energy, carbohydrate, protein and fat intake were not associated with *H. pylori* infection.

### Anthropometric indicators

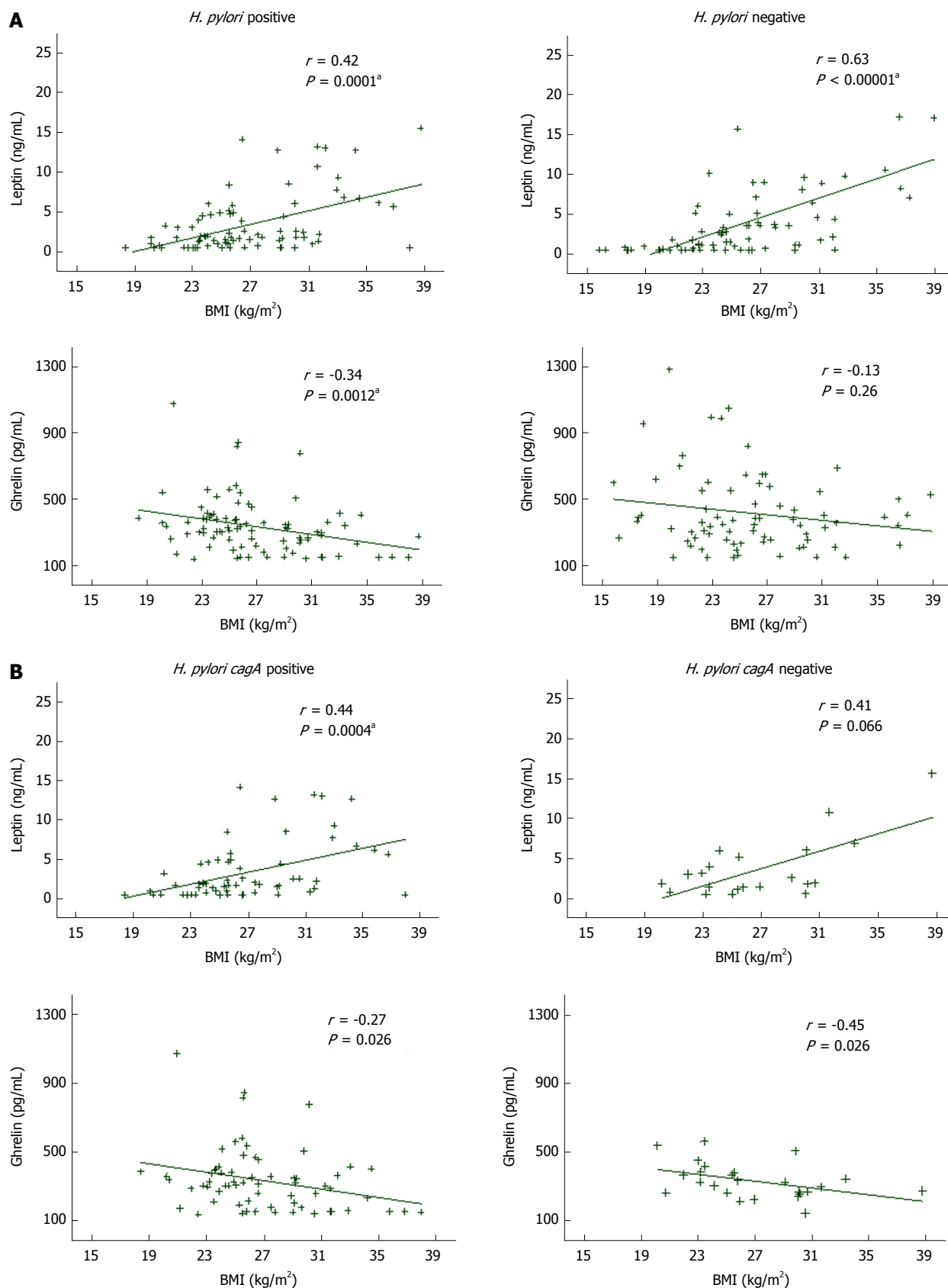
Anthropometric measurements in *H. pylori* positive and negative patients are described in Table 2. BMI of the patients did not differ significantly according to *H. pylori* status (*P* = 0.09), and neither did the percentage of patients with abdominal adiposity, denoted by a high waist circumference (47.1% of the infected patients vs 43.4% of the uninfected patients; *P* = 0.63).

Anthropometric indicators of nutritional status showed that 30/87 (34.5%; 95%CI: 25.3%-44.9%) of the *H. pylori* positive patients had under/normo-weight, 35/87 (40.2%; 95%CI: 30.6%-50.7%) overweight, and 22/87 (25.3%; 95%CI: 17.3%-35.3%) obesity, whereas 38/76 (50.0%; 95%CI: 39.0%-61.0%) of the *H. pylori* negative patients had under/normo-weight, 24/76 (31.6%; 95%CI: 22.2%-42.7%) overweight and 14/76 (18.4%; 95%CI: 11.3%-28.6%) obesity, showing no statistically significant differences between the infected and the uninfected group (*P* = 0.13).

### Ghrelin and leptin concentrations

Appetite hormones serum concentrations are summarized in Table 2. Both ghrelin and leptin serum levels were found to be higher in females than in males (*P* = 0.020 and *P* < 0.0001). Statistical analysis demonstrated that the infection was associated with lower serum ghrelin concentrations (*P* = 0.025), remaining associated after adjusting for BMI and gender in a linear regression analysis (*P* = 0.03). On the other hand, leptin levels did not differ significantly between the infected and the uninfected group (*P* = 0.51), even after adjusting for BMI and gender (*P* = 0.10).

Figure 1 illustrates the correlation between appetite hormones serum concentrations and BMI in the whole population and according to *H. pylori* status. Serum leptin values positively correlated with BMI in the total population (*r* = 0.52; *P* < 0.00001), remaining correlated in the *H. pylori* negatives (*r* = 0.63; *P* < 0.00001) and the *H. pylori* positives (*r* = 0.42; *P* = 0.0001), in which the correlation was obtained only for infected patients carrying *H. pylori* *cagA* positive strains (*r* = 0.44; *P* = 0.0004). In contrast, a weak



**Figure 1** Relationship between leptin and ghrelin serum concentrations and body mass index according to: *Helicobacter pylori* status (A) and *Helicobacter pylori cagA* genotype of infected patients (B). R-values represent Spearman Rank coefficients in multivariate linear regressions, and P values were calculated based on the regressions. <sup>a</sup>Statistically significant (Spearman correlation). BMI: Body mass index.



**Table 3** Type of gastric pathology in *Helicobacter pylori* positive and negative patients

Type of gastric pathology	<i>Helicobacter pylori</i> positive (%)	<i>Helicobacter pylori</i> negative (%)	P value
Antrum	n = 82	n = 70	
Normal	0.0	78.6	< 0.0001 <sup>a</sup>
Chronic inactive gastritis	4.9	12.8	
Chronic active gastritis	78.0	5.7	
Atrophy or intestinal metaplasia	17.1	2.9	
Corpus	n = 77	n = 58	
Normal	2.6	77.6	< 0.0001 <sup>a</sup>
Chronic inactive gastritis	9.1	8.6	
Chronic active gastritis	84.4	6.9	
Atrophy or intestinal metaplasia	3.9	6.9	

<sup>a</sup>P < 0.05, statistically significant (Chi-squared test).

inverse correlation was found between serum ghrelin values and BMI in the whole population ( $r = -0.23$ ;  $P = 0.0036$ ) and in the *H. pylori* positive group ( $r = -0.34$ ;  $P = 0.0012$ ), while a lack of correlation was obtained for ghrelin values and BMI in uninfected patients ( $r = -0.13$ ;  $P = 0.26$ ). When the results of the infected group were analyzed by *cagA* genotype, ghrelin levels remained inversely correlated with BMI both in the *cagA* positive ( $r = -0.27$ ;  $P = 0.026$ ) and the *cagA* negative group ( $r = -0.45$ ;  $P = 0.026$ ) (Figure 1).

#### Gastric pathology, *H. pylori* infection and appetite hormones concentrations

Results of histopathology analysis of gastric biopsies are presented in Table 3. Histological analysis from gastric biopsies could not be performed in 11/163 (6.7%) antrum samples and 28/163 (17.2%) body samples. Presence of *H. pylori* was associated with the type of gastric pathology both in the antrum ( $P < 0.0001$ ) and the corpus ( $P < 0.0001$ ), with a higher prevalence of active chronic gastritis among *H. pylori* positive patients, as has been widely described<sup>[34]</sup>. Table 4 summarizes the results obtained from the analysis of serum ghrelin concentrations according to the type of gastric pathology of the antrum and the corpus in all the patients, independently of their *H. pylori* status. The type and severity of gastric pathology in the corpus were associated with lower serum ghrelin levels ( $P = 0.04$ ). The Kruskal Wallis post-hoc analysis revealed that ghrelin levels differed significantly among all the types of gastric pathology in the corpus except in the chronic inactive and active gastritis groups. On the other hand, gastric pathology of the antrum was not associated with ghrelin levels ( $P = 0.08$ ).

#### *H. pylori* genotype, ghrelin serum levels and BMI

The distribution of *H. pylori vacA* and *cagA* genotypes from infected patients were as follows: The *vacA* S1 allele was detected in 77.0% of the *H. pylori* positive patients, while the *vacA* S2 allele was amplified in 23.0% of the positive group. Overall prevalence of *H.*

*pylori cagA* positive genotype in the antrum and the corpus was 74.7% (95%CI: 64.4%-82.8%). From 62 *H. pylori vacAS1* gastric biopsies, 53 (85.5%) were *cagA* positive and 9 (14.5%) were *cagA* negative both in the antrum and the corpus; while from 18 *H. pylori vacAS2* antrum gastric biopsies and 19 corpus gastric biopsies, 5 (27.8%) and 8 (42.1%) were *cagA* positive, whereas 13 (72.2%) and 11 (57.9%) were *cagA* negative. Due to the association found in this study between *H. pylori* infection and lower ghrelin serum levels, we investigated whether ghrelin levels of infected patients differed according to their *cagA* genotype (Table 5). Although a tendency towards lower ghrelin levels could be observed from antrum and corpus *cagA* positive patients, differences with *cagA* negative patients did not reach statistical significance ( $P = 0.50$  and  $P = 0.49$ , respectively). On the other hand, no statistically significant difference was obtained for the BMI of infected patients carrying a *cagA* negative genotype from those carrying *cagA* positive strains in the gastric antrum ( $P = 0.94$ ) or corpus ( $P = 0.65$ ).

## DISCUSSION

The relationship between *H. pylori* infection and hormonal modulation of food intake, although lately investigated, is still controversial. We conducted this study to evaluate the presence of an association, in dyspeptic patients, between persistent *H. pylori* infection, the genotype of infecting strain, the type of gastric pathology, and the serum ghrelin and leptin levels in patients with a measured anthropometric nutritional status. Our study demonstrated that *H. pylori* infected patients had lower serum ghrelin concentrations than the uninfected ones, independently of their BMI or gender, supporting the findings from several groups<sup>[12-14,17,35]</sup>. It has been demonstrated that ghrelin exhibits gastroprotective antioxidant and anti-inflammatory effects<sup>[36-38]</sup>, modulating gastric mucosal inflammation induced by *H. pylori* lipopolysaccharide

**Table 4** Serum ghrelin levels according to gastric pathology independently of *Helicobacter pylori* status

Type of gastric pathology	Ghrelin levels (pg/mL) <sup>1</sup>				P value
	Normal	Chronic inactive gastritis	Chronic active gastritis	Atrophy/intestinal metaplasia	
Antrum	365.5 (256.0-594.0)	334.5 (223.3-368.0)	306.8 (255.3-389.6)	294.0 (182.8-441.1)	0.08
Corpus	334.5 (242.0-549.0)	323.8 (238.5-371.0)	305.0 (234.0-395.5)	161.0 (150.0-289.0)	< 0.04 <sup>a</sup>

<sup>1</sup>median (IQR); <sup>a</sup>Statistically significant (Kruskal Wallis test).**Table 5** Serum ghrelin according to *cagA* genotype of *Helicobacter pylori* infected patients

Gastric location	Ghrelin levels (pg/mL) <sup>1</sup>		P value
	<i>cagA</i> positive	<i>cagA</i> negative	
Antrum	306.50 (191.00-385.50)	325.50 (258.00-382.00)	0.50
Corpus	311.00 (197.00-395.50)	327.25 (267.00-380.75)	0.49

<sup>1</sup>median (IQR).

(LPS) through several mechanisms which were recently reviewed<sup>[39]</sup>. Ghrelin circulating levels were described to rise in response to severe gastric oxidative stress induced during acute gastritis or peptic ulcer disease; however, its concentration decreases concomitantly with injury of the gastric glands<sup>[36]</sup>. Our results of lower ghrelin levels in persistently infected patients are consistent with these findings, and with the ones from other studies<sup>[35,40]</sup>. In contrast, leptin levels were not associated to *H. pylori* infection in our studied population, as reported by other authors<sup>[14]</sup>, but contrary to previous reports that found lower serum leptin levels in infected individuals<sup>[15]</sup>. Despite the lower ghrelin serum concentrations in *H. pylori* positive patients, their food intake and BMI did not differ when compared to uninfected patients. A lack of association between *H. pylori* infection and dietary intake was previously reported by our group in dyspeptic children<sup>[30]</sup>, and by a Japanese group which described a tendency towards higher energy and carbohydrate intake in *H. pylori* seropositive adults but no statistically significant difference in relation to seronegative adults values<sup>[41]</sup>, which is consistent with the findings of the present study. We used the 24-hour dietary recall for dietary assessment, which might limit our results particularly due to the patients' unintentional misreport<sup>[42]</sup>. However, we consider that the complex and multifactorial nature of food intake regulation would be a more accurate explanation for these findings<sup>[9]</sup>. In this way, a study by Carrasco *et al.*<sup>[43]</sup> showed that the greater decrease in ghrelin levels one year after resective bypass was not associated with differences in dietary intake or weight loss at the same time point. The authors suggest that the restriction of the stomach capacity along with other hormonal mechanisms would be more relevant than the decrease of ghrelin levels on food intake and weight loss<sup>[43]</sup>.

Circulating ghrelin and leptin levels were described to be similar between gender<sup>[44]</sup>; however, we found higher hormonal levels in women than in men, as described in previous reports<sup>[16]</sup>. Our results also showed that circulating leptin concentrations positively correlated with BMI of the patients independently of their *H. pylori* status; however, when the results were analyzed according to *H. pylori* genotype, leptin serum levels remained positively correlated with BMI only in infected patients carrying *cagA* positive strains. Such results are coincident with those reported by Roper *et al.*<sup>[15]</sup>. In addition, ghrelin serum levels inversely correlated with BMI of infected patients independently of their *cagA* genotype.

*H. pylori* positivity was associated with lower ghrelin concentrations; however, we were not able to demonstrate a statistically significant difference in hormonal levels according to the *cagA* genotype despite finding a tendency towards lower ghrelin levels in *cagA* positive patients. It should be pointed out this study was powered to detect differences in hormonal concentrations between *H. pylori* infected and uninfected patients. Consequently, the high prevalence of the *cagA* positive genotype in our population (74.7%) and the low number of patients carrying the *vacA* S2 *cagA* positive less frequent allele, along with an insufficient sample size of infected individuals, may have prevented the observation of these differences, if any.

Another important aspect evaluated in this study was the relationship between the type of gastric pathology and appetite hormones level. The results demonstrated that the type and severity of gastric pathology in the corpus were associated with lower ghrelin serum levels, independently of *H. pylori* status. Our findings are similar to the ones of Isomoto *et al.*<sup>[40]</sup>, and to those recently reported by Ichikawa *et al.*<sup>[18]</sup>, which described a decrease in plasma acyl- and desacyl-ghrelin levels according to the severity of atrophy in hemodialysis patients. The location of ghrelin producing cells in the gastric fundus-corpus<sup>[10]</sup> is consistent with these results. The small number of patients with gastric atrophy or intestinal metaplasia in the *H. pylori* positive and negative groups did not allow us to seek an association between ghrelin levels and gastric atrophy according to *H. pylori* status. In addition, the histological evaluation of one biopsy

from each gastric compartment should be also taken into consideration as a biopsy-based sampling error that could limit our results. Future studies should be performed with a different study design or a higher sample size, where the number of patients with this pathological condition would allow the investigation of that relationship.

In conclusion, our study demonstrated that persistent *H. pylori* infection and the severity of gastric pathology of the corpus are associated with lower ghrelin serum concentrations in dyspeptic patients. Future studies are needed to determine if significantly lower ghrelin levels are observed in *cagA* positive patients.

## ARTICLE HIGHLIGHTS

### Research background

The stomach participates in the production of ghrelin and leptin, two important neuroendocrine hormones in food intake modulation. *Helicobacter pylori* (*H. pylori*) infection has been associated with several pathologies affecting the gastroduodenal mucosa, for which reason it would be important to find out whether it could alter circulating levels of these hormones and ultimately, the body mass index (BMI).

### Research motivation

Although the influence of *H. pylori* infection on the hormonal regulation of food intake has been addressed lately, the results are controversial.

### Research objectives

The present study aimed to evaluate the relationship between *H. pylori* infection, *cagA* genotype, type of gastric pathology, serum ghrelin and leptin concentrations and nutritional status in patients with gastrointestinal symptoms.

### Research methods

This cross-sectional study included fasted dyspeptic adults (18-70 y) referred for an upper digestive endoscopy. We conducted a survey for sociodemographic variables evaluation and a 24 h dietary recall for food intake estimation. *H. pylori* status was determined by three methods: histological analysis, PCR amplification of the *vacA* constitutive *H. pylori* gene and <sup>13</sup>C-Urea Breath Test. Total ghrelin and leptin serum concentrations were measured by enzyme-linked immunosorbent assay and enzyme amplified sensitivity immunoassay respectively. During an upper gastrointestinal endoscopy, four gastric biopsies were obtained. One sample of each gastric site was used for histological assessment and the others for PCR amplification of *H. pylori vacA* and *cagA* genes. Statistical analysis was performed using  $\chi^2$ , Mann-Whitney U, Kruskal-Wallis tests, Spearman's correlation and linear regression.

### Research results

Prevalence of persistent *H. pylori* infection was 53.4% (95%CI: 45.7%-65.8%) in our population of 163 adults. Mean age was  $40.8 \pm 14.0$  years, and 98 (60.1%) were female. Nutrient intake did not differ significantly between *H. pylori* positive and negative patients, neither did BMI. We observed significantly lower serum ghrelin levels in infected patients [median 311.0 pg/mL (IQR 230.0-385.5)] than in uninfected ones [median 355.0 pg/mL (IQR 253.8-547.8)] ( $P = 0.025$ ), even after adjusting for BMI and gender ( $P = 0.03$ ). A tendency towards lower ghrelin levels could be detected from antrum and corpus *cagA* positive patients; however, differences with *cagA* negative patients did not reach statistical significance ( $P = 0.50$  and  $P = 0.49$ , respectively). Lower serum ghrelin concentration was associated with the type and severity of gastric pathology in the corpus ( $P = 0.04$ ), independently of *H. pylori* status. Serum leptin levels did not differ significantly between *H. pylori* positive and negative patients [median

1.84 ng/mL (0.80-4.85) vs 1.84 ng/mL (0.50 - 5.09), ( $P = 0.51$ )].

### Research conclusions

Our study demonstrated that *H. pylori* infection and the severity of gastric pathology of the corpus are associated with lower ghrelin serum concentrations. We also observed lower, but not significantly different ghrelin levels in patients carrying *cagA* positive strains, an observation that should be evaluated further in future studies.

### Research perspectives

Our conclusions highlight the importance of investigating the effect of *H. pylori* eradication on ghrelin circulating levels regarding the genotype of infecting strains.

## ACKNOWLEDGMENTS

We thank the medical team of the Esophagus-Stomach Section, Endoscopy and Pathology Units and the administrative personnel of the Hospital de Gastroenterología "Dr. Carlos Bonorino Udaondo" for their cooperation with our protocol. Our thanks are also due to Dr. Roger A Feldman, who has kindly revised this manuscript.

## REFERENCES

- 1 Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315 [PMID: 6145023]
- 2 Schistosomes, liver flukes and *Helicobacter pylori*. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. *IARC Monogr Eval Carcinog Risks Hum* 1994; **61**: 1-241 [PMID: 7715068]
- 3 Czinn SJ. *Helicobacter pylori* infection: detection, investigation, and management. *J Pediatr* 2005; **146**: S21-S26 [PMID: 15758899 DOI: 10.1016/j.jpeds.2004.11.037]
- 4 Blaser MJ, Atherton JC. *Helicobacter pylori* persistence: biology and disease. *J Clin Invest* 2004; **113**: 321-333 [PMID: 14755326 DOI: 10.1172/JCI20925]
- 5 Atherton JC, Blaser MJ. Coadaptation of *Helicobacter pylori* and humans: ancient history, modern implications. *J Clin Invest* 2009; **119**: 2475-2487 [PMID: 19729845 DOI: 10.1172/JCI38605]
- 6 Frenck RW Jr, Clemens J. *Helicobacter* in the developing world. *Microbes Infect* 2003; **5**: 705-713 [PMID: 12814771]
- 7 Bado A, Levasseur S, Attoub S, Kermorgant S, Laigneau JP, Bortoluzzi MN, Moizo L, Lehy T, Guerre-Millo M, Le Marchand-Brustel Y, Lewin MJ. The stomach is a source of leptin. *Nature* 1998; **394**: 790-793 [PMID: 9723619 DOI: 10.1038/29547]
- 8 Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999; **402**: 656-660 [PMID: 10604470 DOI: 10.1038/45230]
- 9 Konturek PC, Konturek JW, Cześnikiewicz-Guzik M, Brzozowski T, Sito E, Konturek SJ. Neuro-hormonal control of food intake: basic mechanisms and clinical implications. *J Physiol Pharmacol* 2005; **56** Suppl 6: 5-25 [PMID: 16340035]
- 10 Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 2000; **141**: 4255-4261 [PMID: 11089560 DOI: 10.1210/endo.141.11.7757]
- 11 Kojima M, Kangawa K. Ghrelin: more than endogenous growth hormone secretagogue. *Ann N Y Acad Sci* 2010; **1200**: 140-148

- [PMID: 20633142 DOI: 10.1111/j.1749-6632.2010.05516.x]
- 12 **Liew PL**, Lee WJ, Lee YC, Chen WY. Gastric ghrelin expression associated with *Helicobacter pylori* infection and chronic gastritis in obese patients. *Obes Surg* 2006; **16**: 612-619 [PMID: 16687031 DOI: 10.1381/096089206776945002]
  - 13 **Salles N**, Ménard A, Georges A, Salzmann M, de Ledinghen V, de Mascarel A, Emeriau JP, Lamouliatte H, Mégraud F. Effects of *Helicobacter pylori* infection on gut appetite peptide (leptin, ghrelin) expression in elderly inpatients. *J Gerontol A Biol Sci Med Sci* 2006; **61**: 1144-1150 [PMID: 17167154]
  - 14 **Chuang CH**, Sheu BS, Yang HB, Lee SC, Kao AW, Cheng HC, Chang WL, Yao WJ. Gender difference of circulating ghrelin and leptin concentrations in chronic *Helicobacter pylori* infection. *Helicobacter* 2009; **14**: 54-60 [PMID: 19191897 DOI: 10.1111/j.1523-5378.2009.00653.x]
  - 15 **Roper J**, Francois F, Shue PL, Mourad MS, Pei Z, Olivares de Perez AZ, Perez-Perez GI, Tseng CH, Blaser MJ. Leptin and ghrelin in relation to *Helicobacter pylori* status in adult males. *J Clin Endocrinol Metab* 2008; **93**: 2350-2357 [PMID: 18397989 DOI: 10.1210/jc.2007-2057]
  - 16 **Stec-Michalska K**, Malicki S, Michalski B, Peczek L, Wisniewska-Jarosinska M, Nawrot B. Gastric ghrelin in relation to gender, stomach topography and *Helicobacter pylori* in dyspeptic patients. *World J Gastroenterol* 2009; **15**: 5409-5417 [PMID: 19916170 DOI: 10.3748/wjg.15.5409]
  - 17 **Nweneka CV**, Prentice AM. *Helicobacter pylori* infection and circulating ghrelin levels - a systematic review. *BMC Gastroenterol* 2011; **11**: 7 [PMID: 21269467 DOI: 10.1186/1471-230X-11-7]
  - 18 **Ichikawa H**, Sugimoto M, Sakao Y, Sahara S, Ohashi N, Kato A, Sugimoto K, Furuta T, Andoh A, Sakao T, Yasuda H. Relationship between ghrelin, *Helicobacter pylori* and gastric mucosal atrophy in hemodialysis patients. *World J Gastroenterol* 2016; **22**: 10440-10449 [PMID: 28058025 DOI: 10.3748/wjg.v22.i47.10440]
  - 19 **Odenbreit S**, Püls J, Sedlmaier B, Gerland E, Fischer W, Haas R. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science* 2000; **287**: 1497-1500 [PMID: 10688800]
  - 20 **Isomoto H**, Ueno H, Saenko VA, Mondal MS, Nishi Y, Kawano N, Ohnita K, Mizuta Y, Ohtsuru A, Yamashita S, Nakazato M, Kohno S. Impact of *Helicobacter pylori* infection on gastric and plasma ghrelin dynamics in humans. *Am J Gastroenterol* 2005; **100**: 1711-1720 [PMID: 16086706 DOI: 10.1111/j.1572-0241.2005.41492.x]
  - 21 **World Health Organization**. Report of a WHO Expert Committee. Physical Status: The use and interpretation of anthropometry. Geneva: WHO Technical Report Series 854, 1995: 4-33
  - 22 **CDC**. National Health and Nutrition Examination Survey. Anthropometry Procedures Manual. Atlanta: CDC, 2007: 1.1-3.26
  - 23 **World Health Organization**. Report of a WHO Expert Consultation. Waist Circumference and Waist-Hip Ratio. Geneva: World Health Organization, 2008: 1-34
  - 24 **Avila T**, Chiappe C. Atlas Fotográfico de Preparaciones de Alimentos. 1st ed. Buenos Aires: Editorial Akadia, 2010: 1-11
  - 25 **Ministerio de Salud**. Presidencia de la Nación Argentina. SARA: Sistema de Análisis y Registro de Alimentos. Buenos Aires: Ministerio de Salud de la Nación Argentina, 2006 Available from: URL: <http://datos.dinami.gov.ar/produccion/sara/>
  - 26 **Goldman C**, Barrado A, Janjetic M, Balcarce N, Cueto Rua E, Oshiro M, Calcagno ML, Sarrasague MM, Fuda J, Weill R, Zubillaga M, Perez-Perez GI, Boccio J. Factors associated with *H. pylori* epidemiology in symptomatic children in Buenos Aires, Argentina. *World J Gastroenterol* 2006; **12**: 5384-5388 [PMID: 16981273 DOI: 10.3748/wjg.v12.i33.5384]
  - 27 **Janjetic MA**, Goldman CG, Barrado DA, Cueto Rua E, Balcarce N, Mantero P, Zubillaga MB, López LB, Boccio JR. Decreasing trend of *Helicobacter pylori* infection in children with gastrointestinal symptoms from Buenos Aires, Argentina. *Helicobacter* 2011; **16**: 316-319 [PMID: 21762272 DOI: 10.1111/j.1523-5378.2011.00850.x]
  - 28 **Gisbert JP**, Pajares JM. Review article: 13C-urea breath test in the diagnosis of *Helicobacter pylori* infection -- a critical review. *Aliment Pharmacol Ther* 2004; **20**: 1001-1017 [PMID: 15569102 DOI: 10.1111/j.1365-2036.2004.02203.x]
  - 29 **Slater C**, Preston T, Weaver LT. Is there an advantage in normalising the results of the *Helicobacter pylori* [13C]urea breath test for CO2 production rate in children? *Isotopes Environ Health Stud* 2004; **40**: 89-98 [PMID: 15085988 DOI: 10.1080/10256010310001621164]
  - 30 **Janjetic MA**, Mantero P, Cueto Rua E, Balcarce N, Zerbetto de Palma G, Catalano M, Zubillaga MB, Boccio JR, Goldman CG. Dietary and anthropometric indicators of nutritional status in relation to *Helicobacter pylori* infection in a paediatric population. *Br J Nutr* 2015; **113**: 1113-1119 [PMID: 25761510 DOI: 10.1017/S0007114515000483]
  - 31 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20**: 1161-1181 [PMID: 8827022]
  - 32 **van Doorn LJ**, Figueiredo C, Sanna R, Pena S, Midolo P, Ng EK, Atherton JC, Blaser MJ, Quint WG. Expanding allelic diversity of *Helicobacter pylori* vacA. *J Clin Microbiol* 1998; **36**: 2597-2603 [PMID: 9705399]
  - 33 **Akopyants NS**, Clifton SW, Kersulyte D, Crabtree JE, Youree BE, Reece CA, Bukanov NO, Drazek ES, Roe BA, Berg DE. Analyses of the cag pathogenicity island of *Helicobacter pylori*. *Mol Microbiol* 1998; **28**: 37-53 [PMID: 9593295]
  - 34 **Cover TL**. *Helicobacter pylori* Diversity and Gastric Cancer Risk. *MBio* 2016; **7**: e01869-e01815 [PMID: 26814181 DOI: 10.1128/mBio.01869-15]
  - 35 **Kasai C**, Sugimoto K, Moritani I, Tanaka J, Oya Y, Inoue H, Tameda M, Shiraki K, Ito M, Takei Y, Takase K. Changes in plasma ghrelin and leptin levels in patients with peptic ulcer and gastritis following eradication of *Helicobacter pylori* infection. *BMC Gastroenterol* 2016; **16**: 119 [PMID: 27716077 DOI: 10.1186/s12876-016-0532-2]
  - 36 **Suzuki H**, Matsuzaki J, Hibi T. Ghrelin and oxidative stress in gastrointestinal tract. *J Clin Biochem Nutr* 2011; **48**: 122-125 [PMID: 21373264 DOI: 10.3164/jcbn.10-16GFR]
  - 37 **Slomiany BL**, Slomiany A. Induction in gastric mucosal prostaglandin and nitric oxide by *Helicobacter pylori* is dependent on MAPK/ERK-mediated activation of IKK- $\beta$  and cPLA2: modulatory effect of ghrelin. *Inflammopharmacology* 2013; **21**: 241-251 [PMID: 23563696 DOI: 10.1007/s10787-013-0169-5]
  - 38 **Slomiany BL**, Slomiany A. Role of amplification in phospholipase C $\gamma$ 2 activation in modulation of gastric mucosal inflammatory responses to *Helicobacter pylori*: effect of ghrelin. *Inflammopharmacology* 2015; **23**: 37-45 [PMID: 25362585 DOI: 10.1007/s10787-014-0220-1]
  - 39 **Slomiany BL**, Slomiany A. Role of LPS-elicited signaling in triggering gastric mucosal inflammatory responses to *H. pylori*: modulatory effect of ghrelin. *Inflammopharmacology* 2017; **25**: 415-429 [PMID: 28516374 DOI: 10.1007/s10787-017-0360-1]
  - 40 **Isomoto H**, Ueno H, Nishi Y, Yasutake T, Tanaka K, Kawano N, Ohnita K, Mizuta Y, Inoue K, Nakazato M, Kohno S. Circulating ghrelin levels in patients with various upper gastrointestinal diseases. *Dig Dis Sci* 2005; **50**: 833-838 [PMID: 15906753]
  - 41 **Toyonaga A**, Okamatsu H, Sasaki K, Kimura H, Saito T, Shimizu S, Fukuizumi K, Tsuruta O, Tanikawa K, Sata M. Epidemiological study on food intake and *Helicobacter pylori* infection. *Kurume Med J* 2000; **47**: 25-30 [PMID: 10812886]
  - 42 **Willet W**. Issues in analysis and presentation of dietary data. In: Willet W. Nutritional Epidemiology. 2nd ed. New York, NY: Oxford University Press, 1998: 321-346
  - 43 **Carrasco F**, Rojas P, Csendes A, Codoceo J, Inostroza J, Basfi-fer K, Papapietro K, Watkins G, Rojas J, Ruz M. Changes in ghrelin



concentrations one year after resective and non-resective gastric bypass: associations with weight loss and energy and macronutrient intakes. *Nutrition* 2012; **28**: 757-761 [PMID: 22305536 DOI: 10.1016/j.nut.2011.11.004]

- 44 **Ulasoglu C**, Isbilen B, Doganay L, Ozen F, Kiziltas S, Tuncer I. Effect of *Helicobacter pylori* eradication on serum ghrelin and obestatin levels. *World J Gastroenterol* 2013; **19**: 2388-2394 [PMID: 23613634 DOI: 10.3748/wjg.v19.i15.2388]

**P- Reviewer:** Jonaitis LV, Pellicano R, Slomiany BL, Sugimoto M, Ulasoglu C, Xu CF **S- Editor:** Gong ZM **L- Editor:** A **E- Editor:** Huang Y



## Observational Study

# Metal stents placement for refractory pancreatic duct stricture in children

In Sook Jeong, Sung Hee Lee, Seak Hee Oh, Do Hyun Park, Kyung Mo Kim

In Sook Jeong, Sung Hee Lee, Seak Hee Oh, Kyung Mo Kim, Department of Pediatrics, University of Ulsan College of Medicine, Asan Medical Center Children's Hospital, Seoul 05505, South Korea

Do Hyun Park, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, Seoul 05505, South Korea

ORCID number: In Sook Jeong (0000-0002-3094-3603); Sung Hee Lee (0000-0003-3262-9658); Seak Hee Oh (0000-0002-9672-8877); Do Hyun Park (0000-0002-0270-6969); Kyung Mo Kim (0000-0001-7896-6751).

**Author contributions:** Jeong IS, Lee SH, Oh SH and Kim KM contributed to the conception and design of the study; all authors contributed to the study implementation, data acquisition, and data analysis; Jeong IS wrote the manuscript; and all authors contributed to the editing, reviewing, and final approval of the manuscript.

**Institutional review board statement:** This study was reviewed and approved by the Ethics Committee of Asan Medical Center and University of Ulsan College of Medicine (2017-0210).

**Informed consent statement:** Patients were not required to provide informed consent for this study because the analysis used anonymous clinical data that were obtained after each patient had agreed to treatment with written consent.

**Conflict-of-interest statement:** None of the authors have any conflicts of interests to declare.

**Data sharing statement:** There are no additional data available in relation to this manuscript.

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**Manuscript source:** Unsolicited manuscript

**Correspondence to:** Kyung Mo Kim, MD, Professor, Department of Pediatrics, Asan Medical Center Children's Hospital, University of Ulsan College of Medicine, 88, Olympic-ro 43-gil, Songpa-gu, Seoul 05505, South Korea. [kmkim@amc.seoul.kr](mailto:kmkim@amc.seoul.kr)  
Telephone: +82-2-30103380  
Fax: +82-2-4733725

**Received:** November 10, 2017

**Peer-review started:** November 11, 2017

**First decision:** December 13, 2017

**Revised:** December 20, 2017

**Accepted:** December 26, 2017

**Article in press:** December 26, 2017

**Published online:** January 21, 2018

## Abstract

### AIM

To evaluate the use of fully covered self-expandable metal stents (FCSEMSs) for pancreatic duct strictures in children with chronic pancreatitis.

### METHODS

Eight patients with refractory benign dominant stricture of the main pancreatic duct (MPD) were enrolled through chart reviews between December 2014 and June 2017 in a single center. Endoscopic retrograde cholangiopancreatography (ERCP) with placement of a 6-mm FCSEMS with dual flaps was performed. Endoscopic removal of FCSEMSs was performed with a snare or rat-tooth forceps. All procedures were performed by a pediatric gastroenterologist. For the assessment of outcomes, technical and clinical success, adverse events, and stent patency were evaluated retrospectively.

## RESULTS

The placement and removal of the FCSEMSs were successful in all 8 patients. Five patients were boys and 3 were girls. The median age at initial FCSEMS placement was 12 years (range, 5-18 years). The diameters of all the inserted stents were 6 mm, and the lengths were 4-7 cm. The median indwelling time was 6 mo (range, 3-10 mo). No pancreatic sepsis, pancreatitis, cholestasis, or mortality occurred. There was no proximal and distal migration. All subjects showed a patent stent. On follow-up ERCP, the mean diameter of the stricture improved from 1.1 mm to 2.8 mm ( $P < 0.05$ ), whereas that of upstream dilation improved from 8.4 mm to 6.3 mm ( $P < 0.05$ ).

## CONCLUSION

This initial experience showed that temporary FCSEMS placement is feasible and safe for the management of refractory benign MPD stricture in children.

**Key words:** Chronic pancreatitis; Pancreatic duct; Self-expandable metal stent; Child; Endoscopic retrograde cholangiopancreatography

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**Core tip:** This study reports the initial experience with the use of fully covered self-expandable metal stents (FCSEMSs) for recurrent benign pancreatic duct strictures in children. We indwelled 6-mm FCSEMSs with dual flaps in the pancreatic duct of pediatric patients for 6 mo. The placement and removal of the FCSEMSs were technically and clinically successful. There were no adverse events or stent obstruction. The findings support the applicability of FCSEMSs to the pediatric population.

Jeong IS, Lee SH, Oh SH, Park DH, Kim KM. Metal stents placement for refractory pancreatic duct stricture in children. *World J Gastroenterol* 2018; 24(3): 408-414 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i3/408.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i3.408>

## INTRODUCTION

Plastic stents (PSs) or metal stents have been widely used for the management of biliary and pancreatic duct strictures in endoscopic interventions<sup>[1]</sup>. The fully covered self-expandable metal stent (FCSEMS) was designed to compensate for the defects of PSs and uncovered self-expandable metal stents (SEMSs). The SEMS has the advantages of longer patency and resolution of main pancreatic duct (MPD) strictures because its lumen diameter is larger than that of the PS<sup>[2,3]</sup>. Meanwhile, the FCSEMS has been shown to improve tissue embedding by wrapping around the

frame of the uncovered SEMS<sup>[4]</sup>.

As SEMSs were initially used for malignant biliary obstruction<sup>[5]</sup>, FCSEMSs have been widely used for the management of biliary and pancreatic duct strictures in adults<sup>[6]</sup>. FCSEMSs have proven to be a particularly useful intervention for malignant biliary obstruction<sup>[7,8]</sup>, and their use in the management of benign biliary strictures has been expanding steadily<sup>[9]</sup>. In recent studies, FCSEMSs were increasingly used for the management of MPD strictures refractory to PS placement in adults<sup>[10]</sup>.

In general, studies on the application of stents in pediatric patients are lacking. However, most pediatric studies of endoscopic retrograde cholangiopancreatography (ERCP), including our previous study, investigated the use of PSs; therefore, stenting is an important indication in pediatric ERCP<sup>[11-13]</sup>. One pediatric study showed that PS placement was effective for reducing the recurrence of pancreatitis<sup>[14]</sup>.

For these reasons, FCSEMSs may be useful even in pediatric populations. In this study, we evaluated the feasibility, safety, and therapeutic effect of FCSEMSs for the management of benign MPD strictures in pediatric patients with chronic pancreatitis (CP).

## MATERIALS AND METHODS

### Patients

This was a retrospective study of data collected through medical chart reviews between December 2014 and June 2017 at Asan Medical Center Children's Hospital in Seoul, South Korea. Eight patients with CP and benign dominant MPD stricture refractory to PS placement were enrolled (Table 1). Patients with MPD dilatation due to a malignant cause or trauma were excluded. Five patients were boys and 3 were girls. Their median age was 12 years (range, 5-18 years). The patients in this study had pancreatic divisum and/or gene mutations. The genetic mutations were *PRSS1* p.Gly208Ala, *PRSS1* p.Arg122His, *SPINK1* p.Asn34Ser, *SPINK1* p.Thr53Ilefs\*41, *SPINK1* IVS3(+2)T>C, and *CFTR* p.Gln1352His. The strictures were located at the pancreatic head in 5 patients, at the pancreatic neck in 2 patients, and at the pancreatic body in 1 patient. The subjects had a history of previous PS trials after endoscopic sphincterotomy. The median number of days and total duration of PS placement were 2 d (range, 1-7 d) and 57 d (range, 1-431 d), respectively. The stricture was verified using imaging methods [ultrasonography, computed tomography, endoscopic retrograde pancreatography (ERP), or magnetic resonance cholangiography]. A dominant stricture was defined when the contrast medium on the ERP film was not washed out and the upstream MPD dilation was  $\geq 6$  mm in diameter<sup>[15]</sup>.

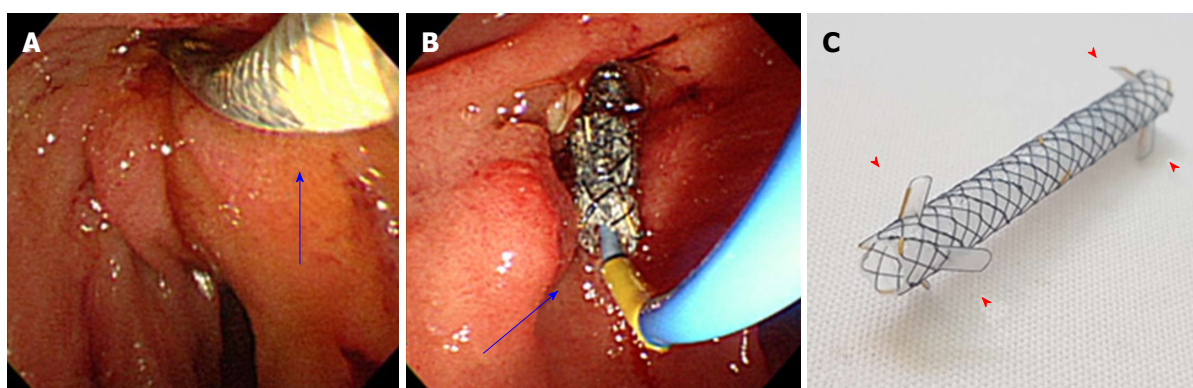
### Procedure

The first session of placement and removal of FCSEMS

**Table 1** Baseline characteristics of the 8 patients with refractory benign dominant main pancreatic duct strictures treated with a fully covered self-expandable metal stent

Case no.	Sex	Anatomical abnormality	Genetic mutation	No. of previous plastic <sup>1</sup> stent placements	Placement time for each plastic stent (d), median (range)	Age at first insertion of a FCSEMS (yr)	Location of the stricture	Diameter and length of the stent (mm/cm)
1	F	Pancreas divisum	<i>PRSS1</i>	2	25.5 (19-32)	17	Head	6/4
2	M	-	<i>SPINK1</i>	7	42.0 (4-93)	12	Head	6/5
3	M	Incomplete pancreas divisum	-	4	126.5 (63-431)	10	Head	6/6
4	M	Pancreas divisum	<i>CFTR</i>	3	57.0 (1-109)	12	Body	6/7
5	F	-	<i>PRSS1</i>	1	46.0	18	Neck	6/7
6	M	-	<i>PRSS1</i>	2	58 (24-92)	5	Head	6/4
7	F	-	<i>SPINK1</i>	1	87	9	Head	6/4
8	M	-	<i>SPINK1</i>	1	29	16	Neck	6/7

<sup>1</sup>Single and multiple stents were used in our study. FCSEMS: Fully covered self-expandable metal stent.



**Figure 1** The fully covered self-expandable metal stent with 3 flaps at both ends used in this study (Hanarostent Biliary dual flap; M.I. Tech, Seoul, South Korea). A and B: The border between the gray and yellow points, shown by the blue arrows, marks the end of the stent. C: Small red arrow heads indicate the flaps.

was performed by an adult and pediatric gastroenterologist. Thereafter, all procedures were performed by a pediatric gastroenterologist. ERP was performed using either an adult duodenoscope (JF; Olympus Optical, Tokyo, Japan) or a therapeutic duodenoscope (TJF, Olympus Optical, Tokyo, Japan). Commercially available 6-mm FCSEMSs (Hanarostent Biliary dual flap; M.I. Tech, Seoul, South Korea) that consisted of nitinol material with a silicone covering membrane were used (Figure 1). After the endoscopic sphincterotomy, which had been described elsewhere, dilatation of the stricture was performed with a Soehendra stent retriever (Wilson Cook Medical, Winston-Salem, NC, United States) and/or a Hurricane dilator balloon (Boston Scientific, Natick, MA, United States) prior to stent placement. The insertion length of the stent was determined such that at least 1 cm of ampulla and normal area at the end of the stenosis were each covered. Repositioning was carried out until the stent was fully inflated and placed in the targeted location. On the basis of previous studies in adults<sup>[16]</sup>, stent removal was planned with rat-tooth forceps at 6 mo after placement, unless adverse events or episodes of pancreatitis had occurred. Informed consent was obtained from the parents of the patients, after the risks and benefits of the procedure and alternative treatments were explained. The study

protocol was approved by the institutional review board of Asan Medical Center (2017-0210).

### Measurement of outcome

Feasibility was evaluated in terms of technical success, migration, and patency. Technical success was determined in accordance with the success of stent placement and removal. Successful stenting was attained when the stent passed through the stricture and the mesh spread easily, followed by a flow of pancreatic juice or washing fluid into the lumen of the stent. Successful removal was attained when the entire stent was removed without ductal leakage during contrast medium injection. Stent patency was assessed *via* visual inspection when the FCSEMS was removed. Efficacy was evaluated according to the improvements of the stricture and reduction of pancreatic pain and pancreatitis. Stricture improvement was evaluated by measuring the diameter of the stricture and the upstream dilation of the MPD as shown on pancreatography after the removal of the first metal stent. Safety was evaluated based on the occurrence of adverse events such as pancreatitis, cholestasis, bowel perforation, sepsis, or mortality. Blood pressure, heart rate, body temperature, laboratory tests (complete blood count and C-reactive protein, total bilirubin, direct bilirubin, amylase, and lipase levels), and abdominal



**Table 2** Resolution of pancreatic duct strictures after the first placement of a fully covered self-expandable metal stent

Case no.	Duration of stenting (mo)	Diameter of the stricture (cm)		Diameter of the dilated segment <sup>1</sup> (cm)	
		Pre	Post	Pre	Post
1	3	0.8	3.0	6.5	4.9
2	10	1.5	2.0	12.1	8.7
3	6	1.2	3.4	11.3	7.1
4	6	0.9	3.4	8.1	6.4
5	6	1.0	4.4	7.1	5.0
6	6	0.9	0.9	6.0	5.2
7	6	1.6	2.1	9.4	6.1
8	6	0.8	3.0	7.0	7.0
mean $\pm$ SD	6 <sup>2</sup>	1.1 $\pm$ 0.3	2.8 $\pm$ 0.9	8.4 $\pm$ 2.5	6.3 $\pm$ 1.6
P value	NA		0.017		0.018

<sup>1</sup>The diameter was measured at the dilated segment upstream of the pancreatic duct stricture; <sup>2</sup>Expressed as a median value. NA: Not applicable.

radiographs were conducted at 3 d and 14–21 d after FCSEMS placement to evaluate for short-term adverse events.

### Statistical analyses

The Wilcoxon signed-rank test was used to compare changes in the diameter of the pancreatic duct between pre- and post-FCSEMS measurements. The IBM SPSS Statistics ver. 23.0 (IBM Co., Armonk, NY, United States) software was used for statistical analysis, and the stenting duration and patency are expressed as median values and ranges. *P* values < 0.05 were considered statistically significant.

## RESULTS

### Evaluation of feasibility

In all 8 patients, the placement of the FCSEMS with dual flaps was technically successful. The diameters of all inserted stents were 6 mm, and the lengths were 4–7 cm. The stents were successfully removed in all patients. The median indwelling time of stenting was 6 mo (range, 3–10 mo; Table 2). One patient had the stent removed 4 mo later than the planned time of removal because of personal reasons. However, stent embedding and occlusion did not develop (Figure 2). The patency of the stent lumen in the 8 patients with an FCSEMS had been maintained upon assessment at the time of stent removal. No stent migrations were observed.

### Evaluation of efficacy

Recurrent pancreatitis and pancreatic pain did not develop in 4 patients during FCSEMS placement. In 1 patient, pain developed 3 mo after stenting, resulting in an early removal that revealed remnant stones blocking the distal MPD, although the lumen of the FCSEMS was patent. Following the removal of the stone and reinsertion of the stent, the pain disappeared and statistically significant improvements in stricture and upstream dilation were observed. The mean stricture diameter was 1.1  $\pm$  0.3 cm before stenting and 2.8  $\pm$  0.9 cm after stenting (*P* < 0.05).

The diameter of the upstream dilatation showed improvement from 8.4  $\pm$  2.5 cm to 6.3  $\pm$  1.6 cm after stenting (*P* < 0.05).

### Safety

No adverse events such as pancreatitis, cholestasis, sepsis, bowel perforation, or mortality occurred during FCSEMS placement (Table 3).

## DISCUSSION

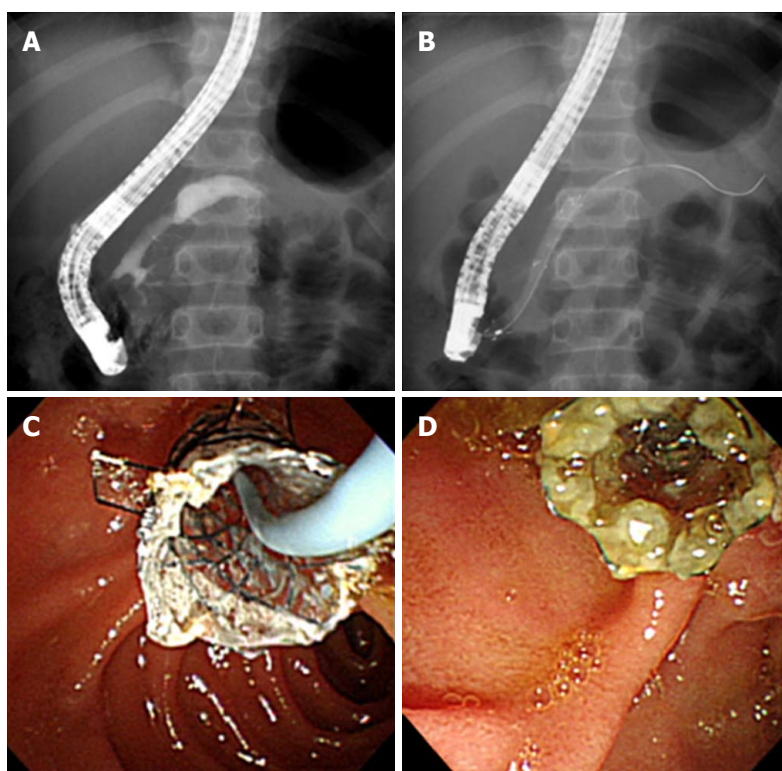
We, herein, report the first case series of the use of FCSEMSs in pediatric patients with CP and MPD strictures refractory to PS placement. Despite the small sample size and lack of long-term follow-up, this preliminary study presents evidence of the feasibility of the use of FCSEMS even in children, with technical and functional success. The disadvantages of PSs include migration and occlusion by sludge, resulting in necessary replacement at around 3 mo<sup>[17]</sup>. In a previous study, FCSEMSs in the management of MPD stricture showed efficacy and safety at 6-mo intervals in adults<sup>[16]</sup>. We suggest that our short-term outcomes are similar to the results for adults in terms of the improvement of strictures and the safety of the procedure<sup>[17]</sup>. Uncovered SEMSs are associated with tissue ingrowth through the stent mesh, which makes their removal difficult<sup>[18]</sup>. Tissue hyperplasia was reported in 23% of patients with partially covered stents<sup>[19]</sup>. When the stent was pulled using rat-tooth forceps at the scheduled removal time, the stent moved easily out of the pancreatic duct without resistance.

Initially, the introduction of FCSEMSs solved the tissue ingrowth issue but led to greater stent migration, with an incidence as high as 41%<sup>[20]</sup>. To overcome this challenge, fins and flaps have been incorporated into these devices<sup>[8,21]</sup>. During our procedure with a dual-flapped FCSEMS, we did not encounter any bleeding, ulcers, or migration problems. In terms of bioavailability, the nitinol materials and silicone membranes of the FCSEMSs applied in the present study series have been used safely in a previous study on the drainage of walled-off necrosis in children<sup>[22]</sup>.

**Table 3** Feasibility and safety of the placement of a fully covered self-expandable metal stent based on the occurrence of adverse events

Case no.	Stent migration	Stent patency	Pancreatitis	Cholestasis	Bowel perforation	Sepsis	Mortality
1	N	Y	N	N	N	N	N
2	N	Y	N	N	N	N	N
3	N	Y	N	N	N	N	N
4	N	Y	N	N	N	N	N
5	N	Y	N	N	N	N	N
6	N	Y	N	N	N	N	N
7	N	Y	N	N	N	N	N
8	N	Y	N	N	N	N	N

N: No; Y: Yes.

**Figure 2** Endoscopic retrograde pancreatography images for case 3. A: Pancreatography image showing a pancreatic duct stricture in the pancreatic neck before the insertion of the fully covered self-expandable metal stent (FCSEMS). B and C: An FCSEMS with flaps placed through the narrow pancreatic duct. D: The stent lumen showing patency at 6 mo after FCSEMS placement.

The major cause of CP in pediatric patients is hereditary pancreatitis rather than gallstones and alcoholism<sup>[23,24]</sup>. Furthermore, because the pancreas reserve of pediatric patients may worsen over time, considering total pancreatectomy with autologous islet transplantation (TP-IAT) may be much more important in children<sup>[25]</sup>. Surgery can result in a reduction of chronic pain through TP-IAT, which was shown to have a more protective effect against negative developments such as postoperative diabetes in preadolescence<sup>[26]</sup>. However, insulin dependency was still present in approximately 40% of postoperative patients<sup>[27]</sup>. Surgical burdens including morbidity, gastric motility dysfunction, and endocrine and exocrine replacement may also occur<sup>[28,29]</sup>. Given that the cause of CP differs between adults and children, adult guidelines for treating this

condition as well as surgical recommendations cannot be automatically applied to pediatric cases. Therefore, until these problems are definitively resolved, endoscopic rather than surgical treatment should be considered in children.

In conclusion, this study showed that the use of FCSEMSs is feasible and safe and may be effective for the management of children with refractory benign MPD strictures. A future long-term investigation with a greater number of patients is warranted.

## ARTICLE HIGHLIGHTS

### Research background

Plastic stents (PSs) or metal stents have been widely used for the management of biliary and pancreatic duct strictures in endoscopic interventions. The fully

covered self-expandable metal stent (FCSEMS) was designed to compensate for the defects of PSs and uncovered self-expandable metal stents (SEMSs). FCSEMSs have been widely used for the management of biliary and pancreatic duct strictures in adults. In general, studies on the application of stents in pediatric patients are lacking. This study reported our experience with using FCSEMS as part of the treatment for benign main pancreatic duct (MPD) strictures in pediatric patients with chronic pancreatitis (CP).

## Research motivation

The major cause of CP in pediatric patients is hereditary pancreatitis rather than gallstones and alcoholism, unlike in adults. Pediatric patients experience mental and physical repetitive pain from complications of MPD stricture during their long life. Stenting is an important indication in pediatric ERCP. In a previous study, the use of FCSEMSs in the management of MPD stricture showed efficacy and safety at 6-mo intervals in adults. We report that our short-term outcomes in children are similar to the results obtained for adults, in terms of the improvement of strictures and the safety of the procedure.

## Research objectives

This study evaluated the feasibility, safety, and therapeutic effect of FCSEMSs for the management of benign MPD strictures in pediatric patients with CP.

## Research methods

This was a retrospective study of data collected through medical chart reviews between December 2014 and June 2017 at Asan Medical Center Children's Hospital in Seoul, Korea. Eight patients with CP and benign dominant MPD stricture refractory to PS placement were enrolled. A dominant stricture was defined when the contrast medium on the endoscopic retrograde pancreatography film was not washed out and the upstream MPD dilation was  $\geq 6$  mm in diameter. Feasibility was evaluated based on technical success, migration, and patency. Technical success was determined in accordance with the success of stent placement and removal. Stricture improvement was evaluated by measuring the diameter of the stricture and the upstream dilation of the MPD. Safety was evaluated based on the occurrence of adverse events at 3 d and 14–21 d after FCSEMS placement. The Wilcoxon signed-rank test was used to compare changes in the diameter of the pancreatic duct between pre- and post-FCSEMS measurements. The IBM SPSS Statistics ver. 23.0 software was used for statistical analysis, and the stenting duration and patency are expressed as median values and ranges.

## Research results

In all 8 patients, the placement of the 6-mm FCSEMS with dual flaps was technically successful. The median indwelling time of stenting was 6 mo (range, 3–10 mo). Stent embedding and occlusion did not develop. The patency of the stent lumen in the 8 patients with an FCSEMS had been maintained upon assessment at the time of stent removal. No stent migrations, adverse events, and mortalities were observed. After the removal of the stone and insertion of the stent, the pain disappeared and statistically significant improvements in stricture and upstream dilation were observed. The mean stricture diameter was  $1.1 \pm 0.3$  cm before stenting and  $2.8 \pm 0.9$  cm after stenting ( $P < 0.05$ ). The diameter of the upstream dilatation showed improvement from  $8.4 \pm 2.5$  cm to  $6.3 \pm 1.6$  cm after stenting ( $P < 0.05$ ). A future study with a larger sample size could clarify the usefulness of FCSEMS in children with benign MPD dilatation.

## Research conclusions

This is the first study to investigate the efficacy and safety of the placement of the 6-mm FCSEMS with dual flaps in children. Despite the technical success and satisfactory clinical outcomes in these 8 children, the present study had certain limitations, including its retrospective design, relatively small sample size, and short follow-up period. In this study, we report our experience with using FCSEMS as part of the management of benign MPD strictures in pediatric patients with CP, providing valuable information about useful treatment for such patients. FCSEMS placement is an effective and safe management strategy for MPD strictures in pediatric patients with CP.

## Research perspectives

We report the first case series of the use of FCSEMSs in the pediatric

population with CP and MPD strictures refractory to PS placement. Despite the small sample size and lack of long-term follow-up, this preliminary study presents evidence of the feasibility of using FCSEMS even in children, with technical and functional success. A future long-term investigation with a greater number of patients is warranted.

## REFERENCES

- 1 **Mangiavillano B**, Pagano N, Baron TH, Arena M, Iabichino G, Consolo P, Opocher E, Luigiano C. Biliary and pancreatic stenting: Devices and insertion techniques in therapeutic endoscopic retrograde cholangiopancreatography and endoscopic ultrasonography. *World J Gastrointest Endosc* 2016; **8**: 143–156 [PMID: 26862364 DOI: 10.4253/wjge.v8.i3.143]
- 2 **Almadi MA**, Barkun A, Martel M. Plastic vs. Self-Expandable Metal Stents for Palliation in Malignant Biliary Obstruction: A Series of Meta-Analyses. *Am J Gastroenterol* 2017; **112**: 260–273 [PMID: 27845340 DOI: 10.1038/ajg.2016.512]
- 3 **Seicean A**, Vultur S. Endoscopic therapy in chronic pancreatitis: current perspectives. *Clin Exp Gastroenterol* 2014; **8**: 1–11 [PMID: 25565876 DOI: 10.2147/CEG.S43096]
- 4 **Shin HP**, Kim MH, Jung SW, Kim JC, Choi EK, Han J, Lee SS, Seo DW, Lee SK. Endoscopic removal of biliary self-expandable metallic stents: a prospective study. *Endoscopy* 2006; **38**: 1250–1255 [PMID: 17163328 DOI: 10.1055/s-2006-944969]
- 5 **Neuhaus H**, Hagenmüller F, Classen M. Self-expanding biliary stents: preliminary clinical experience. *Endoscopy* 1989; **21**: 225–228 [PMID: 2792016 DOI: 10.1055/s-2007-1012954]
- 6 **Hawes RH**. Diagnostic and therapeutic uses of ERCP in pancreatic and biliary tract malignancies. *Gastrointest Endosc* 2002; **56**: S201–S205 [PMID: 12447268 DOI: 10.1067/mge.2002.129003]
- 7 **Eum J**, Park DH, Ryu CH, Kim HJ, Lee SS, Seo DW, Lee SK, Kim MH. EUS-guided biliary drainage with a fully covered metal stent as a novel route for natural orifice transluminal endoscopic biliary interventions: a pilot study (with videos). *Gastrointest Endosc* 2010; **72**: 1279–1284 [PMID: 20870224 DOI: 10.1016/j.gie.2010.07.026]
- 8 **Tsuchiya T**, Itoi T, Gotoda T, Kuraoka K, Sofuni A, Itokawa F, Kurihara T, Ishii K, Tsuji S, Ikeuchi N, Tanaka R, Umeda J, Moriyasu F. A multicenter prospective study of the short-term outcome of a newly developed partially covered self-expandable metallic biliary stent (WallFlex®). *Dig Dis Sci* 2011; **56**: 1889–1895 [PMID: 21298481 DOI: 10.1007/s10620-011-1581-6]
- 9 **Tarantino I**, Mangiavillano B, Di Mitri R, Barresi L, Mocciano F, Granata A, Masci E, Curcio G, Di Pisa M, Marino A, Traina M. Fully covered self-expandable metallic stents in benign biliary strictures: a multicenter study on efficacy and safety. *Endoscopy* 2012; **44**: 923–927 [PMID: 22893134 DOI: 10.1055/s-0032-1310011]
- 10 **Park DH**, Kim MH, Moon SH, Lee SS, Seo DW, Lee SK. Feasibility and safety of placement of a newly designed, fully covered self-expandable metal stent for refractory benign pancreatic ductal strictures: a pilot study (with video). *Gastrointest Endosc* 2008; **68**: 1182–1189 [PMID: 19028228 DOI: 10.1016/j.gie.2008.07.027]
- 11 **Troendle DM**, Barth BA. Pediatric Considerations in Endoscopic Retrograde Cholangiopancreatography. *Gastrointest Endosc Clin N Am* 2016; **26**: 119–136 [PMID: 26616900 DOI: 10.1016/j.giec.2015.08.004]
- 12 **Cho JM**, Jeong IS, Kim HJ, Oh SH, Kim KM. Early adverse events and long-term outcomes of endoscopic sphincterotomy in a pediatric population: a single-center experience. *Endoscopy* 2017; **49**: 438–446 [PMID: 28399609 DOI: 10.1055/s-0043-103956]
- 13 **Agarwal J**, Nageshwar Reddy D, Talukdar R, Lakhtakia S, Ramchandani M, Tandan M, Gupta R, Pratap N, Rao GV. ERCP in the management of pancreatic diseases in children. *Gastrointest Endosc* 2014; **79**: 271–278 [PMID: 24060520 DOI: 10.1016/j.gie.2013.07.060]
- 14 **Oracz G**, Pertkiewicz J, Kierkus J, Dadalski M, Socha J, Ryzko J. Efficiency of pancreatic duct stenting therapy in children with

- chronic pancreatitis. *Gastrointest Endosc* 2014; **80**: 1022-1029 [PMID: 24852105 DOI: 10.1016/j.gie.2014.04.001]
- 15 **Dumonceau JM**, Delhay M, Tringali A, Dominguez-Munoz JE, Poley JW, Arvanitaki M, Costamagna G, Costea F, Devière J, Eisendrath P, Lakhtakia S, Reddy N, Fockens P, Ponchon T, Bruno M. Endoscopic treatment of chronic pancreatitis: European Society of Gastrointestinal Endoscopy (ESGE) Clinical Guideline. *Endoscopy* 2012; **44**: 784-800 [PMID: 22752888 DOI: 10.1055/s-0032-1309840]
- 16 **Ogura T**, Onda S, Takagi W, Kitano M, Sano T, Okuda A, Miyano A, Masuda D, Takeuchi T, Fukunishi S, Higuchi K. Placement of a 6 mm, fully covered metal stent for main pancreatic head duct stricture due to chronic pancreatitis: a pilot study (with video). *Therap Adv Gastroenterol* 2016; **9**: 722-728 [PMID: 27582885 DOI: 10.1177/1756283X16651855]
- 17 **England RE**, Martin DF, Morris J, Sheridan MB, Frost R, Freeman A, Lawrie B, Deakin M, Fraser I, Smith K. A prospective randomised multicentre trial comparing 10 Fr Teflon Tannenbaum stents with 10 Fr polyethylene Cotton-Leung stents in patients with malignant common duct strictures. *Gut* 2000; **46**: 395-400 [PMID: 10673303]
- 18 **Cantù P**, Villa F, Baroni S, Brunati S. Comment to "Precut sphincterotomy, repeated cannulation and post-ERCP pancreatitis in patients with bile duct stone disease". *Dig Liver Dis* 2012; **44**: 625-626 [PMID: 22459567 DOI: 10.1016/j.dld.2012.02.011]
- 19 **Deviere J**, Cremer M, Baize M, Love J, Sugai B, Vandermeeren A. Management of common bile duct stricture caused by chronic pancreatitis with metal mesh self expandable stents. *Gut* 1994; **35**: 122-126 [PMID: 8307432]
- 20 **Deviere J**, Nageshwar Reddy D, Püspök A, Ponchon T, Bruno MJ, Bourke MJ, Neuhaus H, Roy A, González-Huix Lladó F, Barkun AN, Kortan PP, Navarrete C, Peetermans J, Blero D, Lakhtakia S, Dolak W, Lepilliez V, Poley JW, Tringali A, Costamagna G; Benign Biliary Stenoses Working Group. Successful management of benign biliary strictures with fully covered self-expanding metal stents. *Gastroenterology* 2014; **147**: 385-395; quiz e15 [PMID: 24801350 DOI: 10.1053/j.gastro.2014.04.043]
- 21 **Park DH**, Lee SS, Lee TH, Ryu CH, Kim HJ, Seo DW, Park SH, Lee SK, Kim MH, Kim SJ. Anchoring flap versus flared end, fully covered self-expandable metal stents to prevent migration in patients with benign biliary strictures: a multicenter, prospective, comparative pilot study (with videos). *Gastrointest Endosc* 2011; **73**: 64-70 [PMID: 21184871 DOI: 10.1016/j.gie.2010.09.039]
- 22 **Nabi Z**, Lakhtakia S, Basha J, Chavan R, Ramchandani M, Gupta R, Kalapala R, Darisetty S, Talukdar R, Reddy DN. Endoscopic Ultrasound-guided Drainage of Walled-off Necrosis in Children With Fully Covered Self-expanding Metal Stents. *J Pediatr Gastroenterol Nutr* 2017; **64**: 592-597 [PMID: 27977545 DOI: 10.1097/MPG.0000000000001491]
- 23 **Schwarzenberg SJ**, Bellin M, Husain SZ, Ahuja M, Barth B, Davis H, Durie PR, Fishman DS, Freedman SD, Garipey CE, Giefer MJ, Gonska T, Heyman MB, Himes R, Kumar S, Morinville VD, Lowe ME, Nuehring NE, Ooi CY, Pohl JF, Troendle D, Werlin SL, Wilschanski M, Yen E, Uc A. Pediatric chronic pancreatitis is associated with genetic risk factors and substantial disease burden. *J Pediatr* 2015; **166**: 890-896.e1 [PMID: 25556020 DOI: 10.1016/j.jpeds.2014.11.019]
- 24 **Lee YJ**, Kim KM, Choi JH, Lee BH, Kim GH, Yoo HW. High incidence of PRSS1 and SPINK1 mutations in Korean children with acute recurrent and chronic pancreatitis. *J Pediatr Gastroenterol Nutr* 2011; **52**: 478-481 [PMID: 21415673 DOI: 10.1097/MPG.0b013e31820e2126]
- 25 **Chinnakotla S**, Beilman GJ, Dunn TB, Bellin MD, Freeman ML, Radosevich DM, Arain M, Amateau SK, Mallory JS, Schwarzenberg SJ, Clavel A, Wilhelm J, Robertson RP, Berry L, Cook M, Hering BJ, Sutherland DE, Pruett TL. Factors Predicting Outcomes After a Total Pancreatectomy and Islet Autotransplantation Lessons Learned From Over 500 Cases. *Ann Surg* 2015; **262**: 610-622 [PMID: 26366540 DOI: 10.1097/SLA.0000000000001453]
- 26 **Bellin MD**, Carlson AM, Kobayashi T, Gruessner AC, Hering BJ, Moran A, Sutherland DE. Outcome after pancreatectomy and islet autotransplantation in a pediatric population. *J Pediatr Gastroenterol Nutr* 2008; **47**: 37-44 [PMID: 18607267 DOI: 10.1097/MPG.0b013e31815c8af9]
- 27 **Kobayashi T**, Manivel JC, Bellin MD, Carlson AM, Moran A, Freeman ML, Hering BJ, Sutherland DE. Correlation of pancreatic histopathologic findings and islet yield in children with chronic pancreatitis undergoing total pancreatectomy and islet autotransplantation. *Pancreas* 2010; **39**: 57-63 [PMID: 19745778 DOI: 10.1097/MPA.0b013e3181b8ff71]
- 28 **Bhayani NH**, Enomoto LM, Miller JL, Ortenzi G, Kaifi JT, Kimchi ET, Staveley-O'Carroll KF, Gusani NJ. Morbidity of total pancreatectomy with islet cell auto-transplantation compared to total pancreatectomy alone. *HPB (Oxford)* 2014; **16**: 522-527 [PMID: 23992021 DOI: 10.1111/hpb.12168]
- 29 **Radomski M**, Zureikat AH. Total pancreatectomy and islet cell autotransplantation: outcomes, controversies and new techniques. *JOP* 2015; **16**: 1-10 [PMID: 25640776 DOI: 10.6092/1590-8577/2892]

P- Reviewer: Fu D S- Editor: Gong ZM L- Editor: A  
E- Editor: Huang Y





## Prospective Study

# Optimization of hepatobiliary phase delay time of Gd-EOB-DTPA-enhanced magnetic resonance imaging for identification of hepatocellular carcinoma in patients with cirrhosis of different degrees of severity

Jian-Wei Wu, Yue-Cheng Yu, Xian-Li Qu, Yan Zhang, Hong Gao

Jian-Wei Wu, Xian-Li Qu, Yan Zhang, Hong Gao, Department of Radiology, Bayi Hospital, Nanjing University of Chinese Medicine, Nanjing 210002, Jiangsu Province, China

Yue-Cheng Yu, Liver Disease Center, Bayi Hospital, Nanjing University of Chinese Medicine, Nanjing 210002, Jiangsu Province, China

ORCID number: Jian-Wei Wu (0000-0002-8131-636X); Yue-Cheng Yu (0000-0003-3480-1829); Xian-Li Qu (0000-0002-0693-5670); Yan Zhang (0000-0001-6911-2826); Hong Gao (0000-0001-5400-6668).

**Author contributions:** Wu JW and Yu YC conceived and wrote the manuscript; Wu JW, Yu YC, Qu XL, Zhang Y and Gao H participated in designing the research, collecting and analyzing the data, and approving the final version.

**Institutional review board statement:** The study was reviewed and approved by the Academic Committee of Bayi Hospital, Nanjing University of Chinese Medicine.

**Informed consent statement:** Written informed consent for the GED-MRI scanning was obtained from all the enrolled patients and controls.

**Conflict-of-interest statement:** All the authors listed above have no potential conflicts of interest relevant to this article to report.

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Manuscript source: Invited manuscript

Correspondence to: Yue-Cheng Yu, MD, PhD, Chief Doctor, Professor, Liver Disease Center, Bayi Hospital, Nanjing University of Chinese Medicine, No. 34, Section 34, Yanggongjing, Qinhuai District, Nanjing 210002, Jiangsu Province, China. [gslycy@163.com](mailto:gslycy@163.com)  
Telephone: +86-25-80864059  
Fax: +86-25-84586476

Received: October 24, 2017  
Peer-review started: October 25, 2017  
First decision: November 21, 2017  
Revised: December 18, 2017  
Accepted: December 26, 2017  
Article in press: December 26, 2017  
Published online: January 21, 2018

## Abstract

### AIM

To optimize the hepatobiliary phase delay time (HBP-DT) of Gd-EOB-DTPA-enhanced magnetic resonance imaging (GED-MRI) for more efficient identification of hepatocellular carcinoma (HCC) occurring in different degrees of cirrhosis assessed by Child-Pugh (CP) score.

### METHODS

The liver parenchyma signal intensity (LPSI), the liver parenchyma (LP)/HCC signal ratios, and the visibility of HCC at HBP-DT of 5, 10, 15, 20, and 25 min (*i.e.*, DT-5, DT-10, DT-15, DT-20, and DT-25) after injection of Gd-EOB-DTPA were collected and analyzed in 73 patients with cirrhosis of different degrees of severity (including 42 patients suffering from HCC) and 18 healthy adult controls.

## RESULTS

The LPSI increased with HBP-DT more significantly in the healthy group than in the cirrhosis group ( $F = 17.361$ ,  $P < 0.001$ ). The LP/HCC signal ratios had a significant difference ( $F = 12.453$ ,  $P < 0.001$ ) among various HBP-DT points, as well as between CP-A and CP-B/C subgroups ( $F = 9.761$ ,  $P < 0.001$ ). The constituent ratios of HCC foci identified as obvious hypointensity (+++), moderate hypointensity (++), and mild hypointensity or isointensity (+/-) kept stable from DT-10 to DT-25: 90.6%, 9.4%, and 0.0% in the CP-A subgroup; 50.0%, 50.0%, and 0.0% in the CP-B subgroup; and 0.0%, 0.0%, and 100.0% in the CP-C subgroup, respectively.

## CONCLUSION

The severity of liver cirrhosis has significant negative influence on the HCC visualization by GED-MRI. DT-10 is more efficient and practical than other HBP-DT points to identify most of HCC foci emerging in CP-A cirrhosis, as well as in CP-B cirrhosis; but an HBP-DT of 15 min or longer seems more appropriate than DT-10 for visualization of HCC in patients with CP-C cirrhosis.

**Key words:** Magnetic resonance imaging; Gd-EOB-DTPA; Hepatobiliary phase; Delay time; Hepatocellular carcinoma; Cirrhosis; Optimization

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**Core tip:** In order to optimize the hepatobiliary phase delay time (HBP-DT) of Gd-EOB-DTPA-enhanced magnetic resonance imaging for more efficient identification of hepatocellular carcinoma (HCC) in cirrhosis of different degrees of severity, we analyzed the signal intensity and ratios between liver parenchyma and HCC, and the percentages of HCC visibility at a series of HBP-DT points in those patients. The severity of cirrhosis was shown to negatively influence HCC visibility, but DT-10 is already enough and more efficient than longer DT to identify HCC in Child-Pugh (CP)-A and CP-B cirrhosis. DT-15 or longer DT seems more appropriate for HCC visibility in patients with CP-C cirrhosis.

Wu JW, Yu YC, Qu XL, Zhang Y, Gao H. Optimization of hepatobiliary phase delay time of Gd-EOB-DTPA-enhanced magnetic resonance imaging for identification of hepatocellular carcinoma in patients with cirrhosis of different degrees of severity. *World J Gastroenterol* 2018; 24(3): 415-423. Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i3/415.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i3.415>

## INTRODUCTION

Gadolinium-ethoxybenzyl-diethylenetriamine penta-acetic acid (Gd-EOB-DTPA, Primovist; Bayer Schering Pharma, Berlin, Germany) is a modern contrast medium

which can be taken mainly by hepatocytes with relative specificity. It has been used more and more frequently in magnetic resonance imaging (MRI) to provide better diagnosis, differential diagnosis, assessment of hepatic function, and imaging of bile ducts in patients with liver-occupying lesions<sup>[1-3]</sup>. Gd-EOB-DTPA-enhanced MRI (GED-MRI) is proven to have marked advantages over contrast-enhanced computed tomography (CT) in finding smaller hepatocellular carcinoma (HCC), especially those with a diameter less than 20 mm, as well as the recurrent HCC after various antitumor treatments<sup>[4,5]</sup>. However, a disadvantage of GED-MRI is that it needs remarkably long time for scanning, in which the hepatobiliary phase delay time (HBP-DT) is usually set at 15 to 20 min or longer<sup>[6,7]</sup>. For the purpose of applying GED-MRI more efficiently and rationally in complex clinical background, it is very necessary to optimize the HBP-DT in different groups of patients, especially in those with liver-occupying lesions under various stages of cirrhosis<sup>[8,9]</sup>. A study reported that an HBP-DT of 10 min after Gd-EOB-DTPA injection was sufficient for hepatic lesion characterization in patients with normal liver function and without cirrhosis<sup>[10]</sup>. However, whether an HBP-DT of 10 min is suitable for patients with liver dysfunction that correlates with cirrhosis is unknown. Another study concluded that an HBP-DT of 15 min was sufficient for patients with mild liver dysfunction classified as grade A of Child-Pugh score (CP-A); on the other hand, an HBP-DT longer than 5 min was meaningless for lesion characterization in patients with moderate or severe liver dysfunction classified as CP-B or CP-C<sup>[11]</sup>. Clinically, these conclusions are worthy of further investigation and discussion. In the current study, we attempted to identify more efficient and practical HBP-DT of GED-MRI for detection of HCC in the context of different grades of cirrhosis.

## MATERIALS AND METHODS

### Patients

Totally, 73 patients (49 males and 24 females) with a median age of 47 years (range, 23-65 years) who suffered from cirrhosis caused by chronic hepatitis B (CHB) were included in this study. The numbers of patients classified as CP-A, B, and C were 43, 25, and 5, respectively. Eighteen healthy adults were selected as controls, with a male-to-female ratio of 1:1 and a median age was 43 years (range, 23-62 years).

Forty-two of the 73 patients with CHB-related cirrhosis were identified as having HCC, of whom 36 were diagnosed by histopathologic examination using samples from hepatectomy ( $n = 26$ ), liver transplantation ( $n = 4$ ), or liver biopsy ( $n = 6$ ). The rest six patients with HCC were diagnosed clinically by the long history of CHB, typical imaging, and a high level of serum alpha-fetoprotein. In the 36 patients with histopathologic examination, moderately to lowly differentiated HCC was confirmed in 34 patients,

moderately differentiated HCC was found in one patient, and the degree of differentiation could not be distinguished in the last patient.

Totally, 47 HCC foci were found in the 42 patients with HCC, and there were 32, 12, and 3 HCC foci in patients with CP-A ( $n = 27$ ), CP-B ( $n = 12$ ), and CP-C ( $n = 3$ ) disease, respectively. The median diameter of these HCC foci was 2.1 cm (range, 0.5–4.4 cm).

### Exclusion criteria

According to the features and mechanism of GED-MRI, patients who had the following status were excluded from this study: (1) the diameter of one HCC focus, or the sum of diameters of all HCC foci was more than 5 cm; (2) there was cancer embolus or thrombus in the portal vein or its main branch; (3) existence of obstructive jaundice; (4) allergy to Gd-EOB-DTPA; (5) acute/subacute-on-chronic liver failure and severe impairment of renal function; and (6) those who could not cooperate well with the MRI operators during GED-MRI.

### Procedure of GED-MRI

After fasting for at least 6 hours and learning how to breathe and breath-hold correctly in accordance with the order of MRI operator, all subjects underwent MRI of the upper abdomen using a 3.0-T scanner (HDxt; GE Medical Systems) with an 8-channel phased array coil. Parallel imaging using the Array Spatial Sensitivity Encoding Technique (ASSET) were done at first, followed by the axial breath-holding three-dimensional fast spoiled gradient-echo (3D FSPGR) T1 weighted imaging, FSE-XL fat-suppression respiratory-triggered T2 weighted imaging, SE/EPI diffuse weighted imaging, and magnetic resonance cholangiopancreatography.

For contrast-enhanced MRI, Gd-EOB-DTPA was injected as a bolus at 0.025 mmol/kg at a rate of 1.0 mL/s, followed by the same volume of physiological saline flush at a rate of 2.0 mL/s. The dynamic and delayed imaging in the HBP at 5, 10, 15, 20, and 25 min (*i.e.*, at DT-5, DT-10, DT-15, DT-20 and DT-25) was sequentially executed after injection of Gd-EOB-DTPA using an axial fat-suppressed liver acceleration volume acquisition (LAVA) sequence (repetition time = 2.8 ms; echo time = 1.2–1.3 ms; flip angle = 11°; frequency bandwidth of 83.33; field of view = 400–480 mm; acquisition matrix = 224 × 224; ASSET3.00PH; slice thickness = 2.6 mm; phase field of view = 1.0; acquisition time = 12–16 s).

### Measurement of liver parenchyma signal intensity

The liver parenchyma signal intensity (LPSI) of the regions of interest from the centers of the left external lobe, left internal lobe, right anterior lobe, and right rear lobe at the level of the porta hepatis, with each area being about 100 mm<sup>2</sup>, was measured on LAVA images at DT-5, DT-10, DT-15, DT-20, and DT-25 after Gd-EOB-DTPA injection. The regions of interest were

away from the vessels, bile ducts, and lesions to avoid the disturbance on signal intensity. The mean value with a standard deviation (mean ± SD) of LPSI was calculated and compared.

### Calculation of signal ratios of LP to HCC foci

The signal intensity of each HCC focus and corresponding adjacent LP at any HBP-DT point was measured, and the signal ratios of LP to each HCC focus at all HBP-DT points were calculated as follows: signal ratio = (mean signal intensity of LP)/(signal intensity of HCC focus).

### Assessment of HCC visualization in patients with different CP scores

The strength of HCC visualization at each HBP-DT point was assessed by naked eyes, and compared among patients with different grades of CP score. The HCC visualization was subjectively divided into three degrees: obvious hypointensity (+++), moderate hypointensity (++), and hypointensity or isointensity (+/-). All the interpretation of HCC visualization and data processing were done by two senior doctors separately. If there was any disagreement between the two doctors, the data would be presented to a third senior doctor and discussed by the three doctors together to reach a final diagnosis.

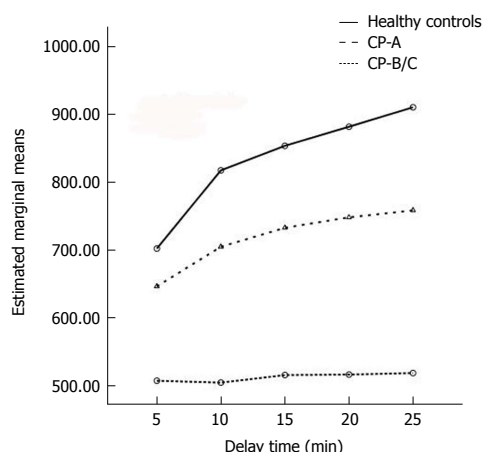
### Statistical analysis

The statistical software SPSS19.0 was used to process the data in this study. Repeated measures two-way analysis of variance (TW-ANOVA) was used to compare the mean LPSI between healthy controls and the cirrhotic group, and the mean LP/HCC signal ratios between subgroups with CP-A cirrhosis and CP-B/C cirrhosis. Repeated measures one-way ANOVA (OW-ANOVA) was used to compare the mean LP/HCC signal ratios at each HBP-DT point in patients suffering from cirrhosis overlapped with HCC, and Huynh-Feldt correction was adopted when the data did not satisfy spherical symmetry.  $P < 0.05$  was regarded as having statistical significance.

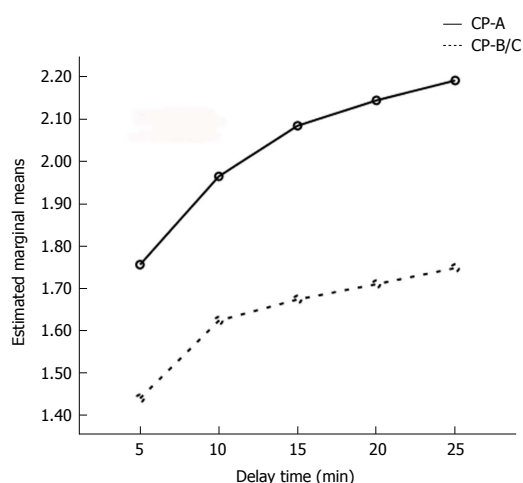
## RESULTS

### Trends of LPSI varying with HBP-DT in healthy controls and patients with cirrhosis

Repeated measures TW-ANOVA and Huynh-Feldt correction showed that the LPSI increased time-dependently in each group included in this study ( $F = 77.109$ ,  $P < 0.001$ ; Figure 1). There was a significant difference for the ascending trends of LPSI among different groups ( $F = 17.361$ ,  $P < 0.001$ ). The strongest LPSI with a markedly ascending trend was seen in healthy controls. Moderate LPSI with a slowly ascending trend was seen in the CP-A subgroup. Mild LPSI without a significantly ascending trend was seen in the CP-B/C subgroup ( $F = 47.685$ ,  $P < 0.001$ ).



**Figure 1** Liver parenchyma signal intensity varying with the hepatobiliary phase delay time of Gd-EOB-DTPA-enhanced magnetic resonance imaging in cirrhotic groups and healthy controls.



**Figure 2** Liver parenchyma/hepatocellular carcinoma signal ratios varying with the hepatobiliary phase delay time of Gd-EOB-DTPA-enhanced magnetic resonance imaging between Child-Pugh (CP)-A and CP-B/C subgroups.

### Correlations between LP/HCC signal ratios and grades of CP score

In the group of HCC coexistent with cirrhosis, there were 32 and 15 HCC foci in the CP-A subgroup and CP-B/C subgroup, respectively. Repeated measures TW-ANOVA and Huynh-Feldt correction showed that the LP/HCC signal ratios increased with HBP-DT in both of the two subgroups ( $F = 8.201$ ,  $P < 0.006$ ; Figure 2), with higher signal ratios in the CP-A subgroup ( $F = 9.761$ ,  $P < 0.001$ ). After DT-10, the curve of signal ratios escalated more slowly in the CP-B/C subgroup than in the CP-A subgroup.

### Comparison of visualization rates of HCC foci at series of HBP-DT points

The MRI images obtained at DT-5 were not assessed for visualization, because much Gd-EOB-DTPA had detained in hepatic vessels and extracellular space at this moment. Accordingly, some of the HCC foci at DT-5

usually appeared as mild hypointensity or isointensity, and thus were difficult for naked eyes to identify.

The visualization rates of HCC foci at DT-10, DT-15, DT-20, and DT-25 kept invariable in the same subgroup (Table 1). On the other hand, there was a significant difference between the CP-A subgroup and CP-B/C subgroup for the constituent ratios of HCC visualization at any corresponding HBP-DT point (Table 1). The constituent ratios of obvious hypointensity (+++) and moderate hypointensity (++) were 90.6% and 9.4% in the CP-A subgroup (Figure 3), 50.0% and 50.0% in the CP-B subgroup (Figure 4), respectively, and there were no HCC foci with mild hypointensity or isointensity (+/-) in both of the two subgroups. It was worth noting that two HCC foci from two patients with CP-C cirrhosis showed only a little increase of visualization with the extension of HBP-DT (Figure 5), and another HCC focus from another patient with CP-C cirrhosis kept the status of isointensity at any HBP-DT point.

## DISCUSSION

Gd-EOB-DTPA is a kind of hepatocyte-specific contrast medium for enhanced MRI, which combines the advantages of both routine MRI contrast media and hepatocyte-specific contrast media. Compared to routine contrast-enhanced CT or MRI scan, GED-MRI could provide not only more accurate diagnosis and differential diagnosis for space-occupying lesions<sup>[12-16]</sup>, early HCC, and hyperplastic nodules of the liver<sup>[17,18]</sup>, but also more accurate assessment of liver function in clinic<sup>[19,20]</sup>.

The key mechanism for Gd-EOB-DTPA to identify HCC is to create a detectable signal contrast between LP and HCC foci in the HBP. After injection, nearly 50% of Gd-EOB-DTPA are taken by hepatocytes through organic anion transporting polypeptide (OATP) 1B1 and 1B3, which are expressed on the hepatocellular membrane, thus contributing to the increase of LPSI. HCC loci usually appear to be hypointense because such lesions could not take or only absorbed a little Gd-EOB-DTPA, and thus could be identified by naked eyes<sup>[21-23]</sup>.

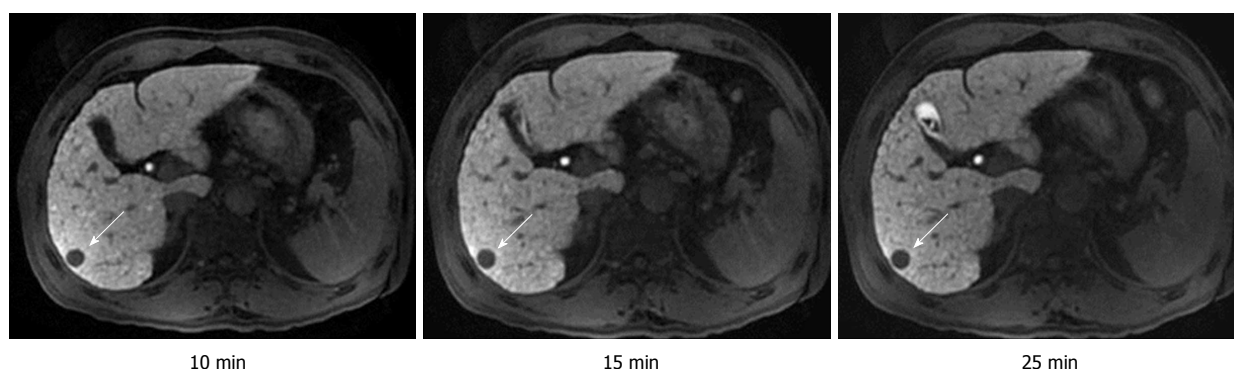
Gd-EOB-DTPA started to be taken by hepatocytes from one and a half minutes after injection, and mounted the peak at about 20 min after injection<sup>[6]</sup>. Some researchers reported that the signal contrast between the liver and spleen reached the peak at a delay time of 60 min, and concluded that it would decrease the contrast of signals between the liver and spleen if the GED-MRI pictures were collected before DT-20 of the HBP<sup>[7]</sup>. However, an important problem is that many patients will feel discomfort if the delay time lasts too long, which makes them difficult to cooperate with operators, and then interferes with the procedure of GED-MRI and decreases its accuracy of diagnosis. Accordingly, it is necessary to optimize, especially



**Table 1** Rates of hepatocellular carcinoma visualization varying with hepatobiliary phase delay time in patients with cirrhosis of different Child-Pugh scores *n* (%)

Grade of CP score	Sum of HCC foci	Signal intensity	HBP-DT				
			5 min	10 min	15 min	20 min	25 min
A	32	+++	0	29 (90.6)	29 (90.6)	29 (90.6)	29 (90.6)
		++	24 (75)	3 (9.4)	3 (9.4)	3 (9.4)	3 (9.4)
		+/-	8 (25)	0	0	0	0
B	12	+++	0	6 (50.0)	6 (50.0)	6 (50.0)	6 (50.0)
		++	7 (58.3)	6 (50.0)	6 (50.0)	6 (50.0)	6 (50.0)
		+/-	5 (41.7)	0	0	0	0
C	3	+++	0	0	0	0	0
		++	0	0	0	0	0
		+/-	3 (100.0)	3 (100.0)	3 (100.0)	3 (100.0)	3 (100.0)

+++; Obvious hypointensity; ++; Moderate hypointensity; +/-; Mild hypointensity or isointensity. CP: Child-Pugh score; HBP-DT: The delay time of the hepatobiliary phase; HCC: Hepatocellular carcinoma.



**Figure 3** Visibility of a hepatocellular carcinoma focus at different hepatobiliary phase delay time points in a patient with Child-Pugh A cirrhosis. An HCC focus of 0.8 cm × 0.8 cm was found in the right rear lobe of the liver in a 53-year-old female patient with cirrhosis caused by chronic hepatitis B. Gd-EOB-DTPA-enhanced magnetic resonance imaging shows a strong contrast between liver parenchyma and HCC focus presenting as obvious hypointensity (+++). The contrast at DT-10 is enough to identify the HCC focus. HCC: Hepatocellular carcinoma.

shorten the scanning time of GED-MRI to a rational extent.

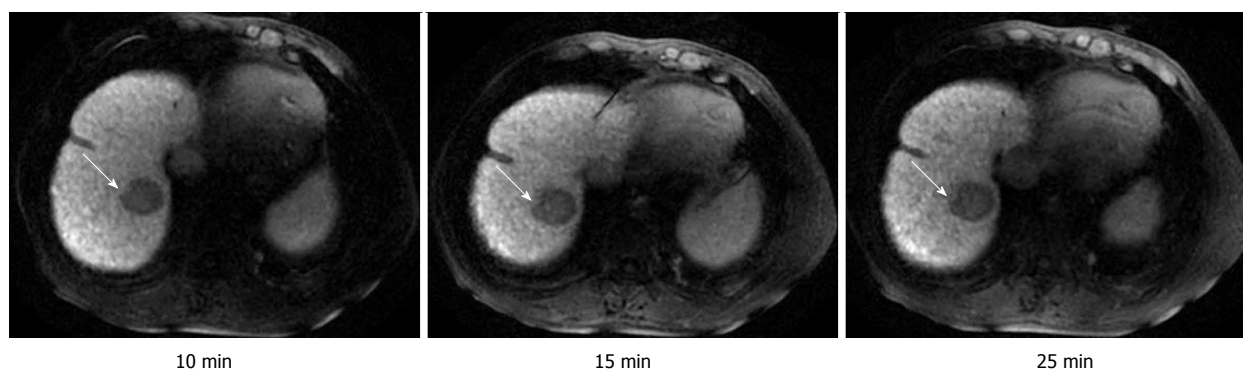
The severity of cirrhosis and liver function impairment could significantly influence the uptake of Gd-EOB-DTPA by LP. Tamada *et al.*<sup>[24]</sup> reported that the LPSI increased significantly during 10 to 20 min of HBP-DT in healthy controls and patients with CP-A or CP-B cirrhosis, but had no significant increase after the portal venous phase. Our investigation showed that the LPSI increased with the time from DT-5 to DT-25 after injection of Gd-EOB-DTPA in healthy controls, but increased relatively slowly in patients with cirrhosis, especially in patients with CP-B/C cirrhosis. This finding suggested that the ability to uptake Gd-EOB-DTPA decreased in liver with cirrhosis, which would lead to a decrease of signal contrast between LP and HCC foci and thus impair the visualization by naked eyes and detection rate of HCC foci.

Most experts believe that 20-30 min of HBP-DT was the best choice for the identification of liver-occupying lesions, but some experts reported that 15 min of HBP-DT was enough to find HCC foci, in spite of less enhancement of LP in CP-C patients than in CP-B or CP-A patients<sup>[17]</sup>. In contrast, our research showed that DT-10 was indeed enough to guarantee

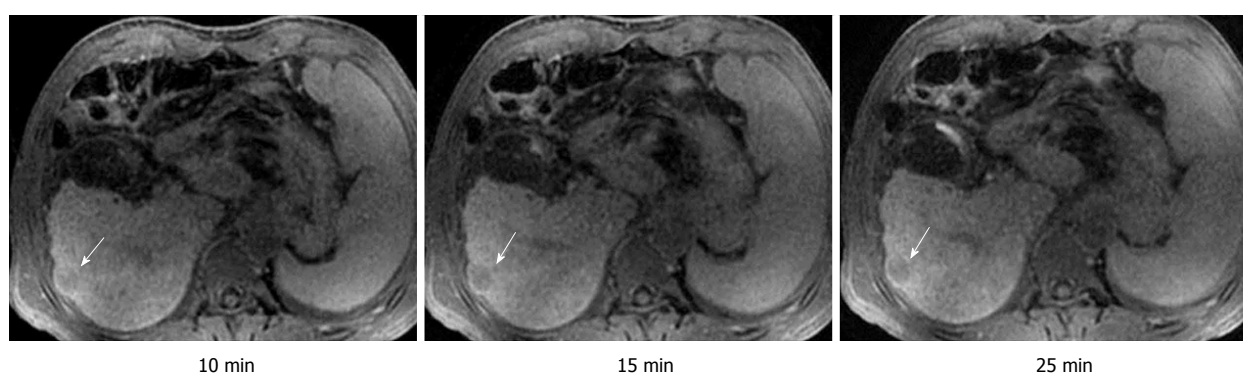
the identification of all HCC foci in 27 patients with CP-A cirrhosis. Although the LP/HCC signal ratios were relatively low in 12 patients with CP-B cirrhosis, all HCC foci were also clearly displayed at DT-10, DT-15, DT-20, and DT-25. These findings showed that DT-10 in GED-MRI could not only sufficiently ensure the identification of HCC foci in patients with CP-A and CP-B cirrhosis, but also markedly shorten the duration of MRI scanning. Although the contrast of signal intensity between LP and HCC foci increased after DT-15, it really had no significant influence on the detection rate of HCC foci. Accordingly, 15 min or longer HBP-DT was not only unnecessary in clinic in patients with CP-A or CP-B cirrhosis, but also decreased the compliance of patients and efficiency of diagnosis during GED-MRI.

The signal contrast was not satisfactory between LP and HCC foci at DT-5, because there was much Gd-EOB-DTPA detained in hepatic vessels, extracellular space, and HCC foci at this time point. Accordingly, although more than half of HCC foci could be visualized at DT-5, we did not recommend images obtained at this time point as the evidence to exclude the diagnosis of HCC.

On the other hand, the signal contrast between LP and HCC foci improved very slowly with time in two



**Figure 4** Visibility of a hepatocellular carcinoma focus at different hepatobiliary phase delay time points in a patient with Child-Pugh B cirrhosis. An HCC focus of 3.6 cm × 3.3 cm was found in the right lobe of the liver in a 58-year-old male patient who was diagnosed with chronic hepatitis B-related cirrhosis. Gd-EOB-DTPA-enhanced magnetic resonance imaging shows a moderate contrast between liver parenchyma and HCC focus presenting as moderate hypointensity (++). The contrast at DT-10 is enough to identify the HCC focus. HCC: Hepatocellular carcinoma.



**Figure 5** Visibility of a hepatocellular carcinoma focus at different hepatobiliary phase delay time points in a patient with Child-Pugh C cirrhosis. An HCC focus of 1.5 cm × 1.7 cm was found in the right lobe of the liver in a 38-year-old male patient who was diagnosed with chronic hepatitis B-related cirrhosis. Gd-EOB-DTPA-enhanced magnetic resonance imaging shows a weak contrast between liver parenchyma and HCC focus presenting as mild hypointensity (+/-). It seems that a longer DT (such as DT-15, DT-20, or DT-25) could slightly improve the visualization of HCC focus. HCC: Hepatocellular carcinoma.

patients with CP-C cirrhosis (See Figure 5), and the signal intensity of HCC focus was nearly equal to that of LP in another patient with CP-C cirrhosis, which made the HCC focus very difficult to be visualized. The poor enhancement of LP at any HBP-DT point in the three patients contributed to the poor signal contrast between LP and HCC foci. These results showed that GED-MRI had no significant advantages over other strategies to identify HCC in patients with CP-C cirrhosis. This conclusion is in accordance with the published findings<sup>[24-26]</sup>. How to optimize the MRI procedure and improve the visualization of HCC foci in patients with CP-C cirrhosis is an important issue awaiting further investigation.

It should be pointed out that many researchers reported that some HCC cells had the ability to take Gd-EOB-DTPA<sup>[27-31]</sup>, but this ability was very poor compared with normal hepatocytes. The ability of HCC foci to take Gd-EOB-DTPA was associated with several factors, including the differentiation degree of HCC and genetic polymorphisms of OATP1B1<sup>[31]</sup>, OATP1B3 (OATP8)<sup>[27-30]</sup>, other OATPs, and hepatocyte nuclear factor 4A (HNF-4A)<sup>[27]</sup>. A few well-differentiated HCCs

could take a bit of Gd-EOB-DTPA, and a very few poorly differentiated HCCs could express OATP1B3 which endowed HCC cells with the ability to take some Gd-EOB-DTPA<sup>[29,30]</sup>. Yamashita *et al.*<sup>[27]</sup> reported that nearly 15% of HCC foci could take Gd-EOB-DTPA in HBP, which was the result of increased expression of OATP1B3 and HNF-4A. Narita *et al.*<sup>[28]</sup> believed that the expression of OATP1B3 was the determinant factor for the uptake of Gd-EOB-DTPA by some HCC foci. Other investigators also reported that the signal intensity and visualization of some HCC foci might be interfered by the ability of HCC cells to take Gd-EOB-DTPA<sup>[29,30]</sup> and the genetic polymorphism of OATP1B1<sup>[31]</sup>. In a recent retrospective study, Miura *et al.*<sup>[32]</sup> reported that 14 patients had high HCC with the LP/HCC signal ratios  $\leq 1.0$ . Clinicopathological analysis revealed low-grade malignancy in high HCC compared with low HCC, and the expression of OATP1B3 was a key mechanism for the hyperintensity in the HBP of GED-MRI. But in our study, we did not find the high HCC lesions except one isointense HCC lesion in a patient with CP-C cirrhosis. It should be noted that borderline lesions of HCC might also show hypo-, iso-, or hyperintensity in the HBP of

GED-MRI, but the hypointense borderline lesions had the highest risk to progress into typical HCC<sup>[33]</sup>.

In summary, our research demonstrated that LPSI in the HBP of GED-MRI was lower in patients with cirrhosis than in healthy controls, but had no significant influence on the visualization rate of HCC foci in patients with CP-A or CP-B cirrhosis. In most of the patients with CP-A or CP-B cirrhosis, DT-10 already satisfied the detection of HCC, and it was not very necessary to prolong the HBP-DT up to 15-25 min. In patients with CP-C cirrhosis, GED-MRI usually had no obvious advantage in detecting HCC; but in some of those cases, a longer HBP-DT of 15-25 min might improve the visualization to a certain extent. Based on the above-mentioned findings and in order to balance the accuracy of diagnosis, efficiency of MRI process, and compliance of the patients, it is advisable to individualize HBP-DT according to the status of liver function for the detection of HCC in patients with cirrhosis. In view of the evidence that HCC foci could take a certain proportion of Gd-EOB-DTPA in some patients, it is very necessary in the future to design larger studies including more cases to analyze the correlation between uptake of Gd-EOB-DTPA and genetic polymorphism or/and expression status of OATP, in order to further clarify the appropriate patients who should receive the examination of GED-MRI and optimize the HBP-DT individually.

## ARTICLE HIGHLIGHTS

### Research background

Gd-EOB-DTPA-enhanced magnetic resonance imaging (GED-MRI) has significant advantages in finding smaller HCC lesions. However, it is believed that GED-MRI may need remarkably long time for scanning, in which the hepatobiliary phase delay time (HBP-DT) is usually set at 15 to 20 min or longer, and many patients could not cooperate well with the operators during the long process of scanning. Recently, a study reported that DT-10 was sufficient for hepatic lesion characterization in patients with normal liver function and without cirrhosis, and another study concluded that DT-15 was sufficient for patients with mild liver dysfunction classified as cirrhosis of Child-Pugh A (CP-A). However, which HBP-DT is both more efficient and more practical for patients with liver dysfunction that correlates with different degrees of severity of cirrhosis is unknown. Accordingly, we attempted to gather new clinical evidence to optimize the HBP-DT of GED-MRI for detection of HCC in the context of different grades of cirrhosis.

### Research motivation

The main topics and key problems of the study include: (1) whether and how the severity of liver cirrhosis will influence the signal intensity of liver parenchyma (LPSI) in the process of GED-MRI examination? (2) whether and how the features of LPSI of liver cirrhosis will interfere with the visibility of HCC? and (3) which HBP-DT will provide the more efficient examination without the discount of diagnostic accuracy in the different context of liver cirrhosis? The findings in the current study will provide first hand information for the answers of above-mentioned questions, and thus contribute to the improvement of rational, efficient, and individual application of GED-MRI.

### Research objectives

This study aimed to optimize the HBP-DT of GED-MRI for more efficient identification of HCC occurring in different degrees of cirrhosis assessed by CP score without any discount of diagnostic accuracy. Through the systematic

assessment of correlations among liver LPSI, liver parenchyma (LP)/HCC signal ratios, and percentage of visibility of HCC lesions at a series of HBP-DT points in the background of liver cirrhosis with different CP scores, it was demonstrated that the HBP-DT of GED-MRI surely could be optimized in the context of liver cirrhosis with different CP scores. Based on those findings and the existence of heterogeneity in the liver function tests, severity of fibrosis, polymorphism of organic anion transporting polypeptide (OATP), and other potential factors that may interfere with the visibility of HCC, a big-sample multicenter prospective study is needed in the future.

### Research methods

This study is a retrospective analysis about how to improve the application of GED-MRI to diagnose HCC occurring in the background of different degrees of cirrhosis. Forty-two patients with HCC from 73 patients with CHB-related cirrhosis were included in this study according to the criteria of inclusion and exclusion. The history and CP scores of CHB-related cirrhosis, the features of LPSI, LP/HCC signal ratios, visibility, and its percentages of HCC lesions at a series of HBP-DT points were systematically collected and compared. The two-way analysis of variance (TW-ANOVA), repeated measures one-way ANOVA (OW-ANOVA), and Huynh-Feldt correction were used to do the statistical analyses of the data. These research methods were the routine ways adopted widely in the clinical investigation.

### Research results

The main findings in this study are as follows. First, the LPSI was found to increase time-dependently both in healthy controls and in patients with HCC overlapped on cirrhosis. Second, the LP/HCC signal ratios had a significant difference among various HBP-DT points, as well as between the CP-A and CP-B/C subgroups. Third, the constituent ratios of HCC foci identified as obvious hypointensity (+++), moderate hypointensity (++), and mild hypointensity/isointensity (+/-) kept stable from DT-10 to DT-25 in each subgroups, but had a difference among subgroups with cirrhosis of CP-A, CP-B, or CP-C. To our knowledge, this is the first time to report that HCC visibility at DT-10 is equal to that at DT-15 or longer DT; that is, compared to longer DT, DT-10 had the same diagnostic accuracy, but showed more efficient diagnosis of HCC existing in the background of both CP-A cirrhosis and CP-B cirrhosis. The problems that remain to be solved in the future include: (1) whether DT-10 will still keep the same high efficiency and accuracy for identification of HCC overlapped on CP-A and CP-B cirrhosis when this concept is applied to more patients; (2) whether and what kind of OATP polymorphism will interfere with the diagnostic efficacy of GED-MRI when used for identification of HCC in Chinese patients; and (3) how to improve the detection of HCC lesions presenting as mild hypointensity or even isointensity in patients with CP-C cirrhosis.

### Research conclusions

The conclusions from this study are summarized as follows. First, the severity of liver cirrhosis has significant negative influence on the HCC visualization by GED-MRI. Second, DT-10 is more efficient and practical than other HBP-DT points to identify most of HCC foci emerging in CP-A cirrhosis, as well as in CP-B cirrhosis. This is the most important new finding of this study. Third, an HBP-DT of 15 min or longer seems more appropriate than DT-10 for visualization of HCC in patients with CP-C cirrhosis. Based on the new findings of this study, we proposed that DT-10 should be chosen as the most appropriate HBP-DT point of GED-MRI for most of the patients with HCC overlapped on CP-A and CP-B cirrhosis, but a longer DT should be used for patients with CP-C patients.

### Research perspectives

This study told clinicians that the status of liver cirrhosis should be assessed carefully before GED-MRI examination in order to choose the most efficient HBP-DT point without any discount of accuracy which is based on the visibility of HCC lesions. It was shown in this study that DT-10 was the optimal HBP-DT which could satisfy the requirement of HCC diagnosis in most of the patients suffering from both CP-A/B liver cirrhosis and HCC, without the necessity of longer HBP-DT. On the other hand, a longer HBP-DT  $\geq 15$  min might improve the visibility of some HCCs overlapped on CP-C cirrhosis. These interesting



findings need to be further confirmed in more patients in the future, and the best method to attain this objective is well-designed multicenter big-sample prospective study.

## REFERENCES

- Ippolito D**, Colombo M, Trattenero C, Bonaffini PA, Talei Franzesi C, Fior D, Sironi S. Diagnostic Value of Semiquantitative Analysis of Dynamic Susceptibility Contrast Magnetic Resonance Imaging with Gd-EOB-DTPA in Focal Liver Lesions Characterization: A Feasibility Study. *Gastroenterol Res Pract* 2015; **2015**: 630273 [PMID: 26064093 DOI: 10.1155/2015/630273]
- Erra P**, Puglia M, Ragozzino A, Maurea S, Liuzzi R, Sabino G, Barbuto L, Cuocolo A, Imbriaco M. Appearance of hepatocellular carcinoma on gadoxetic acid-enhanced hepato-biliary phase MR imaging: a systematic review. *Radiol Med* 2015; **120**: 1002-1011 [PMID: 25900253 DOI: 10.1007/s11547-015-0539-8]
- Xiao YD**, Paudel R, Liu H, Zhang B, Ma C, Zhou SK. Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging: A potential utility for the evaluation of regional liver function impairment following transcatheter arterial chemoembolization. *Oncol Lett* 2015; **9**: 1191-1196 [PMID: 25663880 DOI: 10.3892/ol.2014.2826]
- Saba L**, di Martino M, de Cecco CN, Catalano C, Piga M. Diagnostic confidence of computed tomography and magnetic resonance in focal liver pathology. *Eur J Gastroenterol Hepatol* 2015; **27**: 97-101 [PMID: 25370854 DOI: 10.1097/MEG.0000000000000231]
- Imbriaco M**, De Luca S, Coppola M, Fusari M, Klain M, Puglia M, Mainenti P, Liuzzi R, Maurea S. Diagnostic Accuracy of Gd-EOB-DTPA for Detection Hepatocellular Carcinoma (HCC): A Comparative Study with Dynamic Contrast Enhanced Magnetic Resonance Imaging (MRI) and Dynamic Contrast Enhanced Computed Tomography (CT). *Pol J Radiol* 2017; **82**: 50-57 [PMID: 28217239 DOI: 10.12659/PJR.899239]
- Vogl TJ**, Kümmel S, Hammerstingl R, Schellenbeck M, Schumacher G, Balzer T, Schwarz W, Müller PK, Bechstein WO, Mack MG, Söllner O, Felix R. Liver tumors: comparison of MR imaging with Gd-EOB-DTPA and Gd-DTPA. *Radiology* 1996; **200**: 59-67 [PMID: 8657946 DOI: 10.1148/radiology.200.1.8657946]
- Akimoto S**, Mori H, Fujii T, Furuya K. [Optimal scan timing for Gd-EOB-DTPA enhanced liver dynamic MR imaging]. *Nihon Hoshasen Gijutsu Gakkai Zasshi* 2009; **65**: 626-630 [PMID: 19498252 DOI: 10.6009/jjrt.65.626]
- Kim MY**, Kim YK, Park HJ, Park MJ, Lee WJ, Choi D. Diagnosis of focal liver lesions with gadoxetic acid-enhanced MRI: is a shortened delay time possible by adding diffusion-weighted imaging? *J Magn Reson Imaging* 2014; **39**: 31-41 [PMID: 24115329 DOI: 10.1002/jmri.24122]
- Esterson YB**, Flusberg M, Oh S, Mazzariol F, Rozenblit AM, Chernyak V. Improved parenchymal liver enhancement with extended delay on Gd-EOB-DTPA-enhanced MRI in patients with parenchymal liver disease: associated clinical and imaging factors. *Clin Radiol* 2015; **70**: 723-729 [PMID: 25921617 DOI: 10.1016/j.crad.2015.03.005]
- van Kessel CS**, Veldhuis WB, van den Bosch MA, van Leeuwen MS. MR liver imaging with Gd-EOB-DTPA: a delay time of 10 minutes is sufficient for lesion characterisation. *Eur Radiol* 2012; **22**: 2153-2160 [PMID: 22645040 DOI: 10.1007/s00330-012-2486-2]
- Liang M**, Zhao J, Xie B, Li C, Yin X, Cheng L, Wang J, Zhang L. MR liver imaging with Gd-EOB-DTPA: The need for different delay times of the hepatobiliary phase in patients with different liver function. *Eur J Radiol* 2016; **85**: 546-552 [PMID: 26860666 DOI: 10.1016/j.ejrad.2015.12.015]
- Chou CT**, Chen YL, Wu HK, Chen RC. Characterization of hyperintense nodules on precontrast T1-weighted MRI: utility of gadoxetic acid-enhanced hepatocyte-phase imaging. *J Magn Reson Imaging* 2011; **33**: 625-632 [PMID: 21563246 DOI: 10.1002/jmri.22500]
- Bartolozzi C**, Battaglia V, Bargellini I, Bozzi E, Campani D, Pollina LE, Filippini F. Contrast-enhanced magnetic resonance imaging of 102 nodules in cirrhosis: correlation with histological findings on explanted livers. *Abdom Imaging* 2013; **38**: 290-296 [PMID: 23053453 DOI: 10.1007/s00261-012-9952-9]
- Zhang T**, Lu J, Zhang X, Liang H, Miao X, Jiang J, Ding D, Yang X. [Diagnostic value of hepatobiliary phase imaging with Gd-EOB-DTPA for hepatocellular carcinomas in cirrhosis]. *Zhonghua Yi Xue Za Zhi* 2014; **94**: 517-520 [PMID: 24767294]
- Yoneda N**, Matsui O, Kitao A, Kozaka K, Gabata T, Sasaki M, Nakanuma Y, Murata K, Tani T. Beta-catenin-activated hepatocellular adenoma showing hyperintensity on hepatobiliary-phase gadoxetic-enhanced magnetic resonance imaging and overexpression of OATP8. *Jpn J Radiol* 2012; **30**: 777-782 [PMID: 22911100 DOI: 10.1007/s11604-012-0115-2]
- Fujiwara H**, Sekine S, Onaya H, Shimada K, Mikata R, Arai Y. Ring-like enhancement of focal nodular hyperplasia with hepatobiliary-phase Gd-EOB-DTPA-enhanced magnetic resonance imaging: radiological-pathological correlation. *Jpn J Radiol* 2011; **29**: 739-743 [PMID: 22009428 DOI: 10.1007/s11604-011-0624-4]
- Nakamura S**, Awai K, Utsunomiya D, Namimoto T, Nakaura T, Morita K, Yamashita Y. Chronological evaluation of liver enhancement in patients with chronic liver disease at Gd-EOB-DTPA-enhanced 3-T MR imaging: does liver function correlate with enhancement? *Jpn J Radiol* 2012; **30**: 25-33 [PMID: 22160649 DOI: 10.1007/s11604-011-0003-1]
- Lee NK**, Kim S, Lee JW, Lee SH, Kang DH, Kim GH, Seo HI. Biliary MR imaging with Gd-EOB-DTPA and its clinical applications. *Radiographics* 2009; **29**: 1707-1724 [PMID: 19959517 DOI: 10.1148/rg.296095501]
- Motosugi U**, Ichikawa T, Sou H, Sano K, Tominaga L, Kitamura T, Araki T. Liver parenchymal enhancement of hepatocyte-phase images in Gd-EOB-DTPA-enhanced MR imaging: which biological markers of the liver function affect the enhancement? *J Magn Reson Imaging* 2009; **30**: 1042-1046 [PMID: 19856436 DOI: 10.1002/jmri.21956]
- Yamada A**, Hara T, Li F, Fujinaga Y, Ueda K, Kadoya M, Doi K. Quantitative evaluation of liver function with use of gadoxetate disodium-enhanced MR imaging. *Radiology* 2011; **260**: 727-733 [PMID: 21712472 DOI: 10.1148/radiol.11100586]
- Sahani DV**, Agarwal S, Chung RT. The double-edged sword of functional liver imaging. *Radiology* 2012; **264**: 621-623 [PMID: 22919034 DOI: 10.1148/radiol.12121416]
- Kudo M**. Will Gd-EOB-MRI change the diagnostic algorithm in hepatocellular carcinoma? *Oncology* 2010; **78** Suppl 1: 87-93 [PMID: 20616589 DOI: 10.1159/000315235]
- Chou CT**, Chen YL, Su WW, Wu HK, Chen RC. Characterization of cirrhotic nodules with gadoxetic acid-enhanced magnetic resonance imaging: the efficacy of hepatocyte-phase imaging. *J Magn Reson Imaging* 2010; **32**: 895-902 [PMID: 20882620 DOI: 10.1002/jmri.22316]
- Tamada T**, Ito K, Higaki A, Yoshida K, Kanki A, Sato T, Higashi H, Sone T. Gd-EOB-DTPA-enhanced MR imaging: evaluation of hepatic enhancement effects in normal and cirrhotic livers. *Eur J Radiol* 2011; **80**: e311-e316 [PMID: 21315529 DOI: 10.1016/j.ejrad.2011.01.020]
- Okada M**, Ishii K, Numata K, Hyodo T, Kumano S, Kitano M, Kudo M, Murakami T. Can the biliary enhancement of Gd-EOB-DTPA predict the degree of liver function? *Hepatobiliary Pancreat Dis Int* 2012; **11**: 307-313 [PMID: 22672826 DOI: 10.1016/S1499-3872(12)60165-9]
- Kim JY**, Lee SS, Byun JH, Kim SY, Park SH, Shin YM, Lee MG. Biologic factors affecting HCC conspicuity in hepatobiliary phase imaging with liver-specific contrast agents. *AJR Am J Roentgenol* 2013; **201**: 322-331 [PMID: 23883212 DOI: 10.2214/AJR.12.9478]
- Yamashita T**, Kitao A, Matsui O, Hayashi T, Nio K, Kondo M, Ohno N, Miyati T, Okada H, Yamashita T, Mizukoshi E, Honda M, Nakanuma Y, Takamura H, Ohta T, Nakamoto Y, Yamamoto M, Takayama T, Arai S, Wang X, Kaneko S. Gd-EOB-DTPA-enhanced magnetic resonance imaging and alpha-fetoprotein predict prognosis of early-stage hepatocellular carcinoma. *Hepatology*



- 2014; **60**: 1674-1685 [PMID: 24700365 DOI: 10.1002/hep.27093]
- 28 **Narita M**, Hatano E, Arizono S, Miyagawa-Hayashino A, Isoda H, Kitamura K, Taura K, Yasuchika K, Nitta T, Ikai I, Uemoto S. Expression of OATP1B3 determines uptake of Gd-EOB-DTPA in hepatocellular carcinoma. *J Gastroenterol* 2009; **44**: 793-798 [PMID: 19404564 DOI: 10.1007/s00535-009-0056-4]
- 29 **Kitao A**, Matsui O, Yoneda N, Kozaka K, Shinmura R, Koda W, Kobayashi S, Gabata T, Zen Y, Yamashita T, Kaneko S, Nakanuma Y. The uptake transporter OATP8 expression decreases during multistep hepatocarcinogenesis: correlation with gadoxetic acid enhanced MR imaging. *Eur Radiol* 2011; **21**: 2056-2066 [PMID: 21626360 DOI: 10.1007/s00330-011-2165-8]
- 30 **Kitao A**, Zen Y, Matsui O, Gabata T, Kobayashi S, Koda W, Kozaka K, Yoneda N, Yamashita T, Kaneko S, Nakanuma Y. Hepatocellular carcinoma: signal intensity at gadoxetic acid-enhanced MR Imaging--correlation with molecular transporters and histopathologic features. *Radiology* 2010; **256**: 817-826 [PMID: 20663969 DOI: 10.1148/radiol.10092214]
- 31 **Nassif A**, Jia J, Keiser M, Oswald S, Modess C, Nagel S, Weitschies W, Hosten N, Siegmund W, Kühn JP. Visualization of hepatic uptake transporter function in healthy subjects by using gadoxetic acid-enhanced MR imaging. *Radiology* 2012; **264**: 741-750 [PMID: 22771883 DOI: 10.1148/radiol.12112061]
- 32 **Miura T**, Ban D, Tanaka S, Mogushi K, Kudo A, Matsumura S, Mitsunori Y, Ochiai T, Tanaka H, Tanabe M. Distinct clinicopathological phenotype of hepatocellular carcinoma with ethoxybenzyl-magnetic resonance imaging hyperintensity: association with gene expression signature. *Am J Surg* 2015; **210**: 561-569 [PMID: 26105803 DOI: 10.1016/j.amjsurg.2015.03.027]
- 33 **Kobayashi S**, Matsui O, Gabata T, Koda W, Minami T, Ryu Y, Kozaka K, Kitao A. Relationship between signal intensity on hepatobiliary phase of gadolinium ethoxybenzyl diethylenetriaminepentaacetic acid (Gd-EOB-DTPA)-enhanced MR imaging and prognosis of borderline lesions of hepatocellular carcinoma. *Eur J Radiol* 2012; **81**: 3002-3009 [PMID: 22748558 DOI: 10.1016/j.ejrad.2012.03.029]

**P- Reviewer:** Sakaguchi T   **S- Editor:** Chen K   **L- Editor:** Wang TQ  
**E- Editor:** Huang Y



## Epidemiology of inflammatory bowel disease in racial and ethnic migrant groups

Ravi Misra, Omar Faiz, Pia Munkholm, Johan Burisch, Naila Arebi

Ravi Misra, Naila Arebi, Department of Gastroenterology, St. Marks Academic Institute, London HA1 3UJ, United Kingdom

Omar Faiz, Surgical Epidemiology, Trials and Outcome Centre, St. Marks Academic Institute, London HA1 3UJ, United Kingdom

Pia Munkholm, Johan Burisch, Department of Gastroenterology, North Zealand University Hospital, Frederikssund Frederikssundsvej 30, Denmark

ORCID number: Ravi Misra (0000-0003-0113-9971); Omar Faiz (000369611600046); Pia Munkholm (0000-0003-3325-865X); Johan Burisch (0000-0002-3312-5139); Naila Arebi (0000-0001-6976-1690).

**Author contributions:** Misra R and Arebi N were responsible for the conception and design of the study, and for revising it critically for important intellectual content; Misra R contributed to acquisition of data and analysis; all authors issued final approval of the version to be submitted.

**Conflict-of-interest statement:** There are no conflicts of interest declared.

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**Manuscript source:** Unsolicited manuscript

**Correspondence to:** Naila Arebi, FRCP (Hon), MSc, PhD, Doctor, Department of Gastroenterology, St. Marks Academic Institute, Watford Rd, Harrow, London HA1 3UJ, United Kingdom. [naila.arebi@imperial.ac.uk](mailto:naila.arebi@imperial.ac.uk)  
Telephone: +44-208-8695328  
Fax: +44-208-2354277

Received: August 28, 2017

Peer-review started: August 28, 2017

First decision: October 10, 2017

Revised: November 15, 2017

Accepted: November 21, 2017

Article in press: November 21, 2017

Published online: January 21, 2018

### Abstract

#### AIM

To summarise the current literature and define patterns of disease in migrant and racial groups.

#### METHODS

A structured key word search in Ovid Medline and EMBASE was undertaken in accordance with PRISMA guidelines. Studies on incidence, prevalence and disease phenotype of migrants and races compared with indigenous groups were eligible for inclusion.

#### RESULTS

Thirty-three studies met the inclusion criteria. Individual studies showed significant differences in incidence, prevalence and disease phenotype between migrants or race and indigenous groups. Pooled analysis could only be undertaken for incidence studies on South Asians where there was significant heterogeneity between the studies [95% for ulcerative colitis (UC), 83% for Crohn's disease (CD)]. The difference between incidence rates was not significant with a rate ratio South Asian: Caucasian of 0.78 (95%CI: 0.22-2.78) for CD and 1.39 (95%CI: 0.84-2.32) for UC. South Asians showed consistently higher incidence and more extensive UC than the indigenous population in five countries. A similar pattern was observed for Hispanics in the United States. Bangladeshis and African Americans showed an increased risk of CD with perianal disease.

#### CONCLUSION

This review suggests that migration and race influence

the risk of developing inflammatory bowel disease. This may be due to different inherent responses upon exposure to an environmental trigger in the adopted country. Further prospective studies on homogenous migrant populations are needed to validate these observations, with a parallel arm for in-depth investigation of putative drivers.

**Key words:** Epidemiology; Ethnicity; Migration

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**Core tip:** We reviewed the literature on the epidemiology of inflammatory bowel disease (IBD) in migrants and racial groups. Thirty-three studies met the inclusion criteria. Individual studies showed significant differences in incidence, prevalence and disease phenotype between migrants or race and indigenous groups. Only the incidence studies were sufficient in number and comparable for pooled analysis and meta-analysis. There was a trend for higher incidence for ulcerative colitis and lower incidence for Crohn's disease in South Asian migrants. This review suggests that migration and race influence the risk of developing IBD. This may be due to different inherent responses upon exposure to an environmental trigger in the adopted country.

Misra R, Faiz O, Munkholm P, Burisch J, Arebi N. Epidemiology of inflammatory bowel disease in racial and ethnic migrant groups. *World J Gastroenterol* 2018; 24(3): 424-437 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i3/424.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i3.424>

## INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory bowel conditions, collectively known as inflammatory bowel disease (IBD), the cause of which is unknown. An exaggerated immune response to antigenic stimulation by the gut microbiota on a background of genetic susceptibility is thought to drive the inflammatory process<sup>[1]</sup>.

Epidemiologic studies suggest an increasing incidence and prevalence of IBD in developed countries<sup>[2]</sup>. Although there are fewer epidemiological data from developing countries, there appears to be a similar trend, fuelling its emergence as a global disease<sup>[3,4]</sup>. Some studies reported on a change in migrants moving from developing low incidence countries to developed high incidence countries, whereby they exhibit the incidence of the adopted country<sup>[5,6]</sup>. This phenomenon is worth exploring further for several reasons. Firstly, it implies there may be an environmental trigger for the disease as the onset is too rapid to be accounted for by genetic changes. Secondly, the demographics over the last 50 years have changed due to globalisation

and significant migration to developed countries<sup>[7]</sup>. Disease presentation following migration offers a unique opportunity to further examine how environmental factors might influence disease expression in migrants.

Before undertaking further studies on migrant populations, we sought to summarise the current literature on IBD manifestation after migration to developed countries. Well-recognised large migrant groups have moved from Mexico to the United States and from India to the United States, Europe and the Middle East<sup>[7]</sup>. This environmental change can increase the risk of certain diseases. For example, the Indian migrant group has been studied extensively for cardiovascular disease, with associated significant increased risk<sup>[8,9]</sup>. Migrant communities are largely based on colonial and post-colonial history, cultural and economic ties. When relating to diseases, the distinction between migrants, ethnic group and race is unclear and yet important<sup>[10]</sup>.

The designation of 'migrants' refers to people who move to a new country as a first generation or second generation when born there. Sometimes migrants converge and live within a social community based on historical and cultural ties (e.g., from India or Pakistan to the United Kingdom). The word 'ethnicity' derives from the Greek word *ethnos*, meaning a nation, people or tribe. It represents a multifaceted concept, with emphasis on shared origins or social background, shared cultural traditions and common language. Not all ethnic groups are migrants, instead the term reflects a social categorisation rather than a biological one<sup>[10]</sup>. In contrast the term 'race', first described by Hippocrates over 2000 years ago, classifies man biologically, according to physical characteristics such as face shape and colour. As there may be an overlap between race, ethnicity and migration, we aimed to study the epidemiology of IBD in ethnic migrant and racial groups compared with the indigenous population.

## MATERIALS AND METHODS

This review was registered on the PROSPERO database, with registration number CRD42014013975. A structured search of English language articles in the Medline Ovid database from 1946 to October 4<sup>th</sup>, 2016 was conducted. The Cochrane database was reviewed. The search strategy used the following MeSH headings and key words alone or in combination: inflammatory bowel diseases, Crohn's, ulcerative colitis, epidemiology, incidence, prevalence, diagnosis, migration, race, ethnicity statistics and numerical data (Appendix 1). The reference list of identified studies and reviews were hand-searched and relevant articles included. The search strategy and data extraction was performed by two authors (Misra R and Arebi N). Only published full-text articles were included.

### Study inclusion

(1) Hospital- and population-based studies comparing

incidence and prevalence of UC and/or CD between migrant and indigenous populations; and (2) Studies comparing disease phenotype and disease behaviour by recognised classification between migrant and indigenous populations.

### Data extraction

For each study, data was collected on study design and location; sampling frame; sample size and ethnic group by two authors (Misra R and Arebi N) (Appendices 2 and 3) For incidence studies, the incidence was measured as cases/100000 years. Prevalence was captured as cases per 100000. Phenotype was described using Montreal classification, with E1 to E3 for UC and by age (A1, A2), location (L1, L2, L3, L4) and behaviour (B1, B2, B3, -p) for CD. The proportion of patients with each disease phenotype was expressed as percentage for each group.

### Terminology

The main groups in the studies were described as South Asian (SA), Asian, Hispanic, African-American and Caucasian. SA was defined by persons with a background from the Indian subcontinent: India, Pakistan, Bangladesh and Sri Lanka. Asians were defined by the continent of Asia, encompassing South East Asia and China as well as SA. The term 'migrant' in this review encompassed recently migrated communities, ethnic groups or race. 'Ethnic groups' refers to migrants living as communities (*e.g.*, SA in the United Kingdom, as statistic registration is recorded as an ethnic group). 'Race' refers to migrants settled as mass communities, where over generations they have assimilated with the background communities (Hispanics, African-Americans).

### Statistical analysis

The analysis was restricted to studies comparing the incidence between migrants and Caucasian groups. The incidence of CD and UC were described as rates, and pooled as a rate ratio to compare the incidence between two groups.

The  $\chi^2$  test for heterogeneity was used to determine whether results from different studies varied significantly. Heterogeneity was quantified using the  $I^2$  statistic, to express the percentage of the variability in effect estimates attributed to heterogeneity. The  $P$ -value from the test of heterogeneity is given, with the  $I^2$  value.

As there was significant heterogeneity between the studies, and the  $I^2$  statistic was high for both CD and UC, random effects models were used to assess the size of the difference between population groups.

### Quality assessment

The quality of the incidence and prevalence studies were assessed by whether the diagnostic criteria were clearly defined or recognised criteria were used

(Lennard-Jones and Copenhagen criteria). The method of migrant reporting and sample frame was also examined.

## RESULTS

The PRISMA flow chart is shown in Figure 1. A total of 1181 abstracts were screened. Fifty-two full-text articles were retrieved, and 32 met the inclusion criteria in comparing incidence and prevalence of IBD and disease phenotype and behaviour between migrant and indigenous populations. Only 10 studies were suitable for the meta-analysis.

### Description of incidence and prevalence studies

There were 13 studies identified: 9 measured incidence<sup>[5,6,11-17]</sup> and 4 reported on prevalence<sup>[18-21]</sup> (Tables 1 and 2). Four studies examined both UC and CD<sup>[12,13,18,20]</sup>. Eleven of twelve studies were retrospective, and only one was prospective<sup>[15]</sup>. Ten were conducted in single centres, with only two multicentre studies. Seven studies were carried out in the United Kingdom<sup>[5,6,11,14,15,17,18]</sup>, four in North America<sup>[12,13,19,20]</sup> and the others in Fiji and Singapore<sup>[16,21]</sup>. The prevalence of IBD in the United Kingdom was only reported in one study<sup>[18]</sup>.

### Study quality characteristics

Quality characteristics of the studies are shown in Tables 1, 2 and 3. One study looked at CD and UC incidence, and two studies looked at CD and UC prevalence. Six of thirteen studies used recognised diagnostic criteria<sup>[5,6,12,15,17,21]</sup>. Migrant status was self-reported in five studies<sup>[11,15,18,20,21]</sup> and taken from medical records in six studies<sup>[6,12-14,16,19]</sup>. Two studies relied on patient surname to identify migrant origin<sup>[5,17]</sup>. The sample frame was population-based in six<sup>[5,13,15,17,18,20]</sup> and hospital-based in seven studies<sup>[6,11,12,14,16,19,21]</sup>.

### Incidence of CD

Most studies reporting the incidence of CD (3/5) were from the United Kingdom (Table 1), with a predominant SA migrant group<sup>[6,11,17]</sup>. The remaining two studies from Canada described the SA paediatric population<sup>[12]</sup>, and one study compared non-immigrants to SA<sup>[13]</sup>. The incidence of CD in SAs was consistently lower than Caucasian, except for one Canadian paediatric study<sup>[12]</sup>. The Benchimol study showed a lower incidence in SA compared to other groups within the same environment<sup>[13]</sup>. The two United Kingdom studies where the incidence was examined over two time periods showed an increase in the incidence of CD in the SA population, from 1.2 to 2.3/100000 in East London and 1.2 to 3.1/100000 in Leicester<sup>[6,17]</sup>. The last migrant United Kingdom incidence studies were published in 1989.

### Incidence of UC

There were six studies reporting the incidence of UC (Table 1). The incidence was higher for SAs when



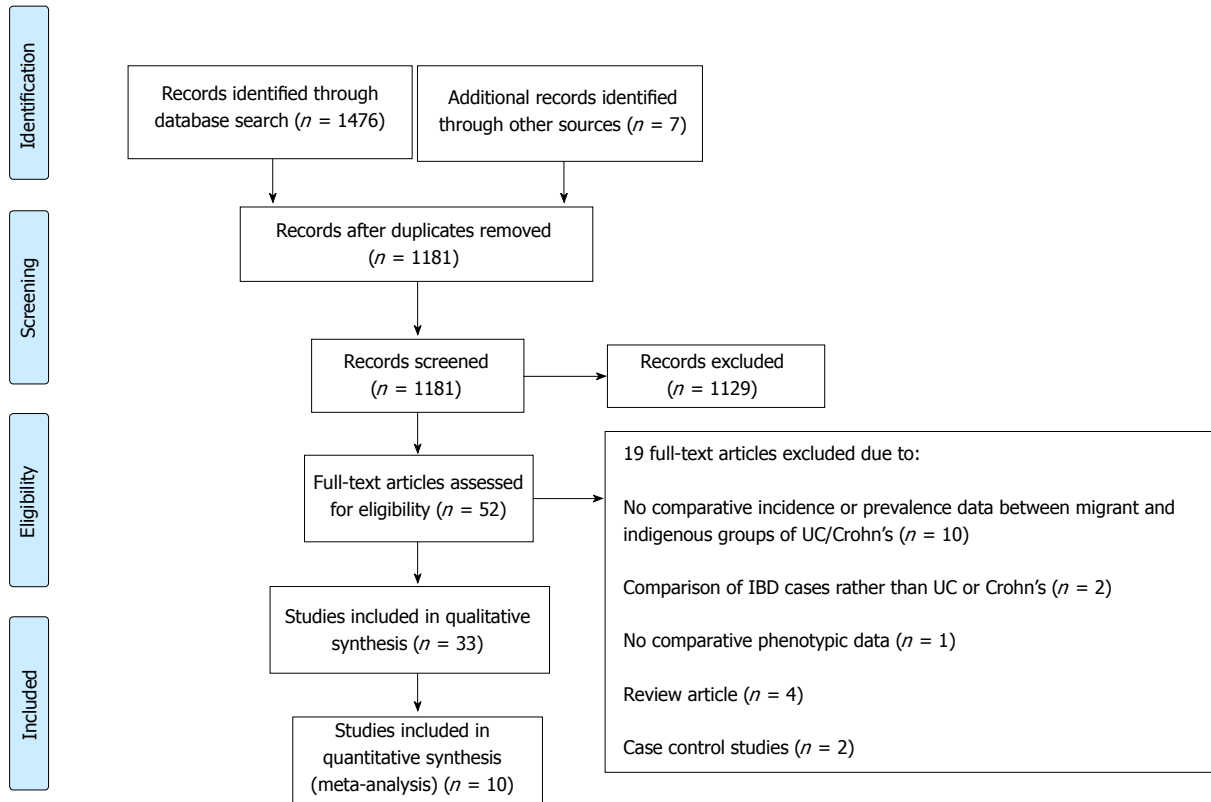


Figure 1 PRISMA flowchart. IBD: Inflammatory bowel disease; UC: Ulcerative colitis.

compared with Caucasians (three studies) and Melanesians in one study from Fiji<sup>[5,12,15,16]</sup>. In the only prospective study, the incidence was significantly higher and although this was a small study (74 cases) it was also the most recent<sup>[15]</sup>. This may indicate a rising incidence. Two studies showed a lower UC incidence, one in the Bangladeshi population in East London<sup>[14]</sup> and the other in Ontario, Canada<sup>[13]</sup>.

#### Meta-analysis of CD incidence studies

We performed a meta-analysis on studies comparing SA migrant and Caucasian groups, which consisted of four studies. Two studies looked at incidence over 2 separate decades. Each decade was counted as a separate study and the analysis was undertaken as six studies<sup>[5,6]</sup>. The Benchimol study<sup>[13]</sup> was excluded as the comparator group and was classified as non-immigrant rather than a specifically named ethnic/migrant group.

The overall rate ratio for CD (0.78, 95%CI: 0.22-2.78) showed a trend towards a lower incidence in the adult SA group in comparison to the Caucasian group (Table 4). The individual studies all showed lower incidence rates, except for one study in a paediatric population which showed a higher incidence in the SA population<sup>[12]</sup>.

#### Meta-analysis of UC incidence studies

Analysis of the four studies for UC showed an overall rate ratio of 1.39 (95%CI: 0.84-2.32), indicating a trend towards a higher rate of UC in the SA population

(Table 4). All but one study reported a higher incidence for UC in SA. In the outlier study<sup>[9]</sup>, the population was exclusively a Bangladeshi subgroup of the SA population, unlike the other studies where the population was predominantly North Indian.

#### Prevalence of CD

Three studies reported on CD prevalence. Two were from the United States, where the prevalence rate for Asian, Black or Hispanic populations was lower than for Caucasians<sup>[19,20]</sup> (Table 2). The remaining study from the United Kingdom showed a lower prevalence in Caucasians than SA compared to Caucasians<sup>[18]</sup>. This is consistent with the reportedly lower SA CD incidence from the same population in Leicester at approximately the same time period<sup>[17]</sup> (Table 1).

#### Prevalence of UC

There were four studies reporting on UC prevalence, with diverging results (Table 4). The only United Kingdom study showed a higher prevalence for UC in SAs compared with the local population<sup>[18]</sup>. A separate study from Singapore reported a higher prevalence in SAs than Chinese and Malay groups<sup>[21]</sup>. In contrast in the United States, the UC prevalence was lower for Asians, Hispanics and Blacks compared with the Caucasian group<sup>[20]</sup>.

#### Description of disease phenotype studies

There were 20 studies examining disease phenotype

**Table 1 Incidence of Crohn's disease and ulcerative colitis**

Study characteristics and demographics					Study quality characteristics			
Study	Country (region)	Study period	Number of cases	Incidence rate/100000		Diagnosis based on recognised criteria	Ethnicity reporting method	Sample frame
				SA	Caucasian			
Crohn's disease								
Fellows (1985) <sup>[11]</sup>	United Kingdom (Derby)	1966-1985	221	4.4	7.5	No	Self-reported	Hospital
Probert (1992) <sup>[6]</sup>	United Kingdom (East London)	1970-79	45	1.2	3.8	Yes	Medical records	Hospital
		1980-89	54	2.3	3.8			
Jayanthi (1992) <sup>[17]</sup>	United Kingdom (Leicester)	1972-1980	80	1.2	3.5	Yes	Surname	Population
		1981-1989	104	3.1	5.3			
Pinsk (2007) <sup>[12]</sup>	Canada (Vancouver)	1985-2005	397 (Paed)	6.7	1.0	Yes	Medical records	Hospital
Benchimol <sup>1</sup> (2015) <sup>[13]</sup>	Canada (Ontario)	1994-2010	12113	5.0	11.3	No	Medical records	Population
Ulcerative colitis								
Probert (1992) <sup>[5]</sup>	United Kingdom (Leicester)	1972-1989	1003	10.8	5.3	No	Surname	Population
Jayanthi (1992) <sup>[14]</sup>	United Kingdom (East London)	1972-1989	112	1.8	6.2	No	Medical records	Hospital
Carr (1999) <sup>[15]</sup>	United Kingdom (Leicester)	1991-1994	74	17.2	9.1	Yes	Self-reported	Population
Pinsk (2007) <sup>[12]</sup>	Canada (Vancouver)	1985-2005	120 (Paed)	6.4	3.7	No	Medical records	Hospital
Probert (1985) <sup>[16]</sup>	Fiji	1985-1986	15	1.5	0.2	No	Medical records	Hospital
Benchimol <sup>1</sup> (2015) <sup>[13]</sup>	Canada (Ontario)	1994-2010	12713	2.0	11.4	No	Medical records	Population

<sup>1</sup>SA compared to non-immigrant. Characteristics of studies comparing South Asian (SA) migrants and Caucasians.

**Table 2 Prevalence of Crohn's disease: Study characteristics and comparison between Caucasian and other migrant groups**

Study characteristics					Study quality characteristics						
Study	Country (region)	Study period	Number of cases	Prevalence rate, cases/100000					Diagnosis based on recognised criteria	Ethnicity reporting method	Sample frame
				SA	Asian	Caucasian	Black	Hispanic			
Probert (1993) <sup>[18]</sup>	United Kingdom (Leicester)	1989	676	33.2	-	75.8	-	-	Yes	Self-reported	Population
Kurata (1992) <sup>[19]</sup>	United States (California)	1982-1988	169	-	5.6	43.6	29.8	4.1	No	Medical records	Hospital
Wang (2013) <sup>[20]</sup>	United States (National Database)	1996-2007	204	-	45.0	154.0	68.0	15.0	No	Self-reported	Population

SA: South Asian.

**Table 3 Prevalence of ulcerative colitis: Study characteristics and comparison between population groups**

Study characteristics					Study quality characteristics						
Study	Country (region)	Study period	Number of cases	Prevalence rate, cases/100000					Diagnosis based on recognised criteria	Ethnicity reporting method	Sample frame
				SA	Asian	Caucasian	Black	Hispanic			
Probert (1993) <sup>[18]</sup>	United Kingdom (Leicester)	1989	888	136.0	-	90.8	-	-	Yes	Self-reported	Population
Wang (2013) <sup>[20]</sup>	United States (National database)	1996-2007	108	-	40.0	89.0	25	35	No	Self-reported	Population
Lee (2000) <sup>[21]</sup>	Singapore (Singapore)	1985-1996	58	SA 16.2	Malay 7.0	Chinese 6.0	Black -	Hispanic -	Yes	Self-reported	Hospital

SA: South Asian.

in relation to migrants or race (Tables 5-9). Fourteen studies were conducted in the United States<sup>[22-34]</sup> and four in the United Kingdom<sup>[15,35-37]</sup>, with the remaining two from Canada and Malaysia<sup>[38,39]</sup>. Sixteen studies were single centre, three multicentre<sup>[24,31,35]</sup> and only one was prospective<sup>[15]</sup>. Majority of the studies reported on both UC and CD, with 15 reporting on UC and 16 on CD.

## CD

The phenotype was studied for location and behaviour. Comparisons are presented for SA, African-Americans or Hispanics and Caucasians depending on the country where the studies were conducted.

SA group was compared with Caucasians in four studies and all reported on both location and disease

**Table 4** Meta-analysis of incidence studies showing rate ratio of South Asians relative to Caucasians

Disease	Number of studies	Heterogeneity		Effect size	
		<i>I</i> <sup>2</sup>	<i>P</i> value	RR (95%CI)	<i>P</i> value
Crohn's disease	6	95%	< 0.001	0.78 (0.22, 2.78)	0.7
Ulcerative colitis	4	83%	0.001	1.39 (0.84, 2.32)	0.2

**Table 5** Crohn's disease location and behaviour

Study	Country (Region)	Time period	Number of cases	Montreal classification	Population groups, %	
					SA	Caucasian
Location						
Walker (2011) <sup>[35]</sup>	United Kingdom (NW London)	2008-2010	309	L1	16.0	24.7
				L2	46.8	34.8
				L3	37.2	40.5
				L4	0.0	0.0
Goodhand (2012) <sup>[36]</sup>	United Kingdom (East London)	2010	141	L1	33.0	32.0
				L2	24.0	43.0
				L3	38.0	22.0
				L4	18.0	13.0
Li (2013) <sup>[22]</sup>	United States (San Francisco)	1994-2009	57 (Paed)	L1	7.7	14.9
				L2	38.5	23.4
				L3	53.9	61.7
				L4	15.4	5.3
Carroll (2016) <sup>[38]</sup>	Canada (British Columbia)	1997-2012	638 (Paed)	L1	6.0	9.0
				L2	55.0	35.0
				L3	39.0	55.0
				L4a	55.0	43.0
				L4b	1.0	9.0
				L4ab	0.0	8.0
Behaviour						
Walker (2011) <sup>[35]</sup>	United Kingdom (NW London)	2008-2010	309	B1	72.3	58.1
				B2	22.0	60.0
				B3	4.3	14.0
				Perianal	20.2	21.4
Goodhand (2012) <sup>[36]</sup>	United Kingdom (East London)	2010	141	B1	63.0	55.0
				B2	15.0	11.0
				B3	20.0	15.0
				Perianal	16.0	3.0
Li (2013) <sup>[22]</sup>	United States (San Francisco)	1994-2009	57 (Paed)	Perianal	46.2	12.8
Carroll (2016) <sup>[38]</sup>	Canada (British Columbia)	1997-2012	638 (Paed)	B1	61.0	73.0
				B2	13.0	16.0
				B3	6.0	3.0
				B2B3	21.0	9.0

South Asians compared with Caucasians. Total No. of cases reported was 1145. Significant difference in disease behaviour illustrated in bold.

behaviour (Table 5). Two were from the United Kingdom<sup>[35,36]</sup>, one from the United States<sup>[22]</sup>, and one from Canada<sup>[38]</sup>. Two studies (United Kingdom and Canada) showed SA significantly more likely to have colonic disease<sup>[35,38]</sup>. These two studies contained 947 cases of the 1147 pooled population. The Canadian study also showed that SA had less ileal involvement<sup>[38]</sup>. There was no other significant difference in disease location. For behaviour, one United Kingdom study showed SA had less stricturing (B2) and less non-penetrating disease (B3), but this was not significant<sup>[35]</sup>. In contrast, perianal disease was significantly more common in SA compared with Caucasians in two studies<sup>[22,36]</sup>. The Canadian paediatric study, which included 638 cases, indicated a more complicated disease behaviour for SA, where B2 and B3 disease was noted in 21% of SA compared with 9% of Caucasians<sup>[38]</sup>.

African-American and Caucasian groups were compared for disease location in eight studies (Table 6), all from the United States, three of which were in paediatric populations<sup>[25,26,29]</sup>. Disease behaviour in eight studies is shown in Table 7. African-Americans showed significantly less ileal disease (L1) in three studies<sup>[23,24,40]</sup> from a population of 2241 cases (65%) out of the total cohort of 3467 cases studied. African-Americans showed more significantly more stricturing (B2) and penetrating disease (B3) in one study<sup>[25]</sup> and perianal disease in two studies<sup>[24,31]</sup> (Table 7).

Hispanic and Caucasians were compared for disease location in only three studies (Table 6) and behaviour in four studies (Table 7). Hispanics showed significantly lower rates for ileal disease and higher rate of colonic disease compared with Caucasians in one study population of 697 cases<sup>[24]</sup>. Perianal presentation was

**Table 6 Crohn's disease location**

Study	Country (Region)	Time period	Number of cases	Disease location/ Behaviour, Montreal	Population groups, %		
					AA	Caucasian (or other control)	Hispanic
Cross (2006) <sup>[23]</sup>	United States (Baltimore)	1997-2005	210	L1	<b>13.0</b>	<b>38.0</b>	-
				L2	29.0	23.0	-
				L3	56.0	36.0	-
Sofia (2014) <sup>[40]</sup>	United States (Chicago)	2008-2013	1334	L1	<b>57.8</b>	<b>71.0</b>	-
				L2	60.6	66.0	-
Nguyen (2006) <sup>[24]</sup>	United States (National)	2003-2005	697	L1	<b>16.1</b>	<b>29.2</b>	23.3
				L2	33.9	17.4	16.3
				L3	22.6	36.7	52.3
				L4	27.4	16.7	8.1
Eidelwein (2007) <sup>[25]</sup>	United States (Baltimore)	1991-2000	137 (Paed)	L1	3.0	2.9	-
				L2	73.5	71.8	-
				L3	23.5	25.2	-
Hatter (2012) <sup>[26]</sup>	United States (Texas)	2004-2009	246 (Paed)	L1	4.3	4.7	4.8
				L2	7.1	4.8	10.5
				L3	87.8	88.9	84.2
Ghazi (2013) <sup>[27]</sup>	United States (Baltimore)	2004-2009	296	L1	31.0	38.0	-
				L2	12.0	20.0	-
				L3	55.0	42.0	-
Damas (2013) <sup>[28]</sup>	United States (Florida)	1998- 2009	325	L1	-	16 (NHW)	24.4
				L2	-	24.0	25.6
				L3	-	60.0	50.0
				L4	-	12.5	3.9
Kugathasan (2003) <sup>[29]</sup>	United States (National)	2000-2001	222 (Paed)	L1	10.0	19.0	-
				L2	34.0	28.0	-
				L3	46.0	51.0	-
				L4	32.0	42.0	-

AA or Hispanics compared with Caucasians (3467 total cases). Bold font shows significant differences between AA and population group. *Italic* font illustrates significant difference between Hispanic and Caucasian groups. AA: African-Americans; NHW: Non-Hispanic white.

significantly more frequent in Hispanics in only 2/4 studies but these studies represented 92% of the studied cohorts (7376/7974)<sup>[24,31]</sup>. In another study of 325 cases, they were significantly less likely to have upper gastrointestinal disease<sup>[28]</sup>.

## UC

The UC studies described disease extent. Results were presented for SA, African-Americans or Hispanics compared with Caucasian or indigenous adult and paediatric populations (Tables 8 and 9). SA and Caucasian (or Malay in one study) groups were compared in six studies, including four in the United Kingdom<sup>[15,35-37]</sup> and one in Malaysia<sup>[39]</sup> and a further study in Canada which was limited to a paediatric population<sup>[38]</sup> (Table 8). In all six studies there were more SA with pan-colonic disease than Caucasians; this was a significant finding in four studies<sup>[35,36,38,39]</sup>, which represented 898/1054 cases (85%) of the pooled population.

African-Americans were compared to Caucasians in six studies, of which two were paediatric (Table 9). Four studies described a higher proportion of African-Americans having disease limited to the rectum<sup>[25,26,32,33]</sup>, which was a significant finding in one study<sup>[33]</sup>.

Hispanics were compared with Caucasians in four studies. In the Hispanic group, two studies showed no difference in disease location for UC<sup>[26,28]</sup>, another

significantly less proctitis<sup>[32]</sup>, with a fourth study demonstrating a significantly increased risk of pan-colitis and higher colectomy rate (32.3% vs 15.8%,  $P < 0.01$ )<sup>[24]</sup>.

## DISCUSSION

This review indicates a difference in disease incidence, prevalence and phenotypes between migrants and non-migrants in individual studies. This has two important implications. Firstly, increased migration may alter disease burden within a population and impact health policy decisions. Secondly, deeper investigation of migrant populations may provide insight into the role of environmental change and diet in the aetiology of IBD.

We identified the main migrant populations with IBD reported in the literatures as SA, African-Americans and Hispanics. The SA population has a wide diaspora and countries such as United Kingdom, Canada, Singapore, Malaysia and Fiji report the presentation of IBD.

The SA group seems to be particularly susceptible to developing UC, with a higher incidence than the local population in the United Kingdom and Canada. The incidence was only lower in two populations. The first was in east London, which was restricted to the Bangladeshi group and therefore may be a reflection of a distinct ethnic group. The second was in Ontario, Canada, which compared SA to the non-



**Table 7 Crohn's disease behaviour**

Study	Country (Region)	Time period	Number of cases	Disease location/ Behaviour, Montreal	Population groups, %		
					AA	Caucasian	Hispanic
Nguyen (2006) <sup>[24]</sup>	United States (National)	2003-2005	697	B1	53.2	48.0	45.3
				B2	27.9	21.4	23.6
				B3	19.0	30.6	31.1
				<b>Perianal</b>	<b>40.0</b>	<b>28.7</b>	52.5
Eidelwein (2007) <sup>[25]</sup>	United States (Baltimore)	1991-2000	137 (Paed)	<b>B2 + B3</b>	<b>29.1</b>	<b>11.11</b>	-
Ghazi (2013) <sup>[27]</sup>	United States (Baltimore)	2004-2009	296	B1	18.0	33.0	-
				B2	42.0	36.0	-
				B3	40.0	31.0	-
				Perianal	36.0	25.0	-
Sofia (2014) <sup>[40]</sup>	United States (Chicago)	2008-2013	1334	Perianal	25.7	24.7	-
Damas (2013) <sup>[28]</sup>	United States (Florida)	1998- 2009	325	B1	-	81.5 (NHW)	78.9
				B2	-	0.0	1.1
				B3	-	18.5	20.0
				Perianal	-	27.0	23.0
Kugathasan (2005) <sup>[29]</sup>	United States (National)	-	222 (Paed)	Inflammatory	58.0	64.0	-
				Stricturing	20.0	18.0	-
				Fistulising	22.0	18.0	-
				Perianal	23.0	36.0	-
Malaty (2010) <sup>[30]</sup>	Houston (Texas)	2000-2006	273	Inflammatory	64.0	63.0	58.0
				Stricturing	16.0	17.0	13.0
				Fistulising	17.0	16.0	32.0
Adler (2016) <sup>[31]</sup>	Multicentre (United States and United Kingdom)	2006-2014	2034 (Paed)	<b>Perianal</b>	<b>26.0</b>	<b>20.0</b>	24.0

AA or Hispanics compared with Caucasians (5318 cases). *Italic font* illustrates significant differences between hispanic and population group. **Bold font** shows significant differences between AA and other population group. AA: African-Americans.

**Table 8 Ulcerative colitis in Caucasian and South Asian migrant groups (1054 cases)**

Study	Country (Region)	Study period	Number of cases	Disease extent, Montreal	Ethnic groups, %	
					SA	Caucasian (or other control)
Adult						
Carr (1999) <sup>[15]</sup>	United Kingdom (Leicester)	1991-1994	74	E1 + E2	63.7	68.3
				E3	36.3	31.7
Rashid (2008) <sup>[37]</sup>	United Kingdom (Manchester)	2008	82	E3	41.0	26.0
Walker (2011) <sup>[35]</sup>	United Kingdom (NW London)	2008-2010	461	E1	9.9	26.1
				E2	27.1	31.4
				E3	63.0	42.0
Goodhand (2012) <sup>[36]</sup>	United Kingdom (East London)	2010	89	E1	2.0	31.0
				E3	60.0	33.0
Hilmi (2009) <sup>[39]</sup>	Malaysia (Malaysia)	2004-2005	118	E1 + 2	39.6	56.7 (Malay)
				E3	60.4	43.4 (Malay)
Paediatric						
Carroll (2016) <sup>[38]</sup>	Canada (British Columbia)	1997-2012	230	E1	3.0	5.0
				E2	9.0	19.0
				E3	12.0	18.0
				E4	77.0	58 (Non-South Asian)

Adult and paediatric studies on disease extent. **Bold font** shows significant differences between AA and population group (809/1054 cases). AA: African-American; SA: South Asian.

immigrant population<sup>[13]</sup>. The non-immigrant population had a higher UC incidence rate (11.4/100000) than the Caucasian group in the other Canadian study (3.7/100000). A possible explanation is that immigration data was only available from 1985 onwards. Immigrants who arrived to Canada before 1985 were considered as non-immigrants. It is noteworthy that the likelihood of UC increased the younger age of arrival of the immigrant.

Whilst there were fewer studies on prevalence,

both reported higher prevalence for SA as would be expected for the higher incidence rate<sup>[18,21]</sup>. When the data was pooled, the summary measures also illustrated a trend towards a higher rate of UC in the SA population compared to Caucasians (Table 4). The outlier study consisted of a Bangladeshi ethnic group, and excluding this study showed a consistently higher incidence of UC in SA; but, we did not repeat the meta-analysis on the three remaining studies as the result would be questionable, due to bias and error.

**Table 9** Ulcerative colitis in AA and Hispanic groups (1787 cases)

Study	Country (Region)	Time period	Number of cases	Disease extent	Ethnic groups, %		
					AA	Caucasian	Hispanic
Adult studies							
Basu (2005) <sup>[32]</sup>	United States (Texas)	1999-2003	61	E1	-	32.0	8.0
				E2 + 3	-	68.0	86.0
Nguyen (2006) <sup>[24]</sup>	United States (National database)	2003-2005	396	E1	13.8	5.9	3.6
				E2	16.4	31.4	34.5
				E3	51.7	62.7	80.0
Eidelwein (2007) <sup>[25]</sup>	United States (Baltimore)	1991-2000	40	E1 + 2	0.0	10.0	-
				E3	100.0	23.0	-
Moore (2012) <sup>[33]</sup>	United States (Ohio)	2000-2010	311	E1	14.5	6.5	-
				E2	44.7	30.7	-
				E3	40.8	62.9	-
Sofia (2014) <sup>[40]</sup>	United States (Chicago)	2008-2013	541	E1	17.9	11.1	-
				E2	28.6	32.0	-
				E3	50.0	51.5	-
Damas (2013) <sup>[28]</sup>	United States (Florida)	1999-2009	138	E1	-	11.8	12.5
				E2	-	29.4	46.2
				E3	-	58.8	41.3
Paediatric							
Flasar (2008) <sup>[34]</sup>	United States (Baltimore)	1997-2005	197	E1	23.0	10.0	-
				E2	23.0	31.0	-
				E3	53.0	59.0	-
Hatter (2012) <sup>[26]</sup>	United States (Texas)	2004-2009	103	E1 + 2	7.1	15.4	18.8
				E3	81.3	84.6	81.3

Adult and paediatric studies on disease extent. *Italic* font illustrates significant difference between Hispanic and Population group. Bold font shows significant differences between AA and population group. AA: African-American.

Data on African-Americans and Hispanics was limited for disease incidence and prevalence, and no conclusions could be drawn. Only one study from the United States described UC prevalence in African-Americans and Hispanics, which was lower for each group than the Caucasian population<sup>[20]</sup>. Two separate disease phenotype studies in Hispanics reported less proctitis in one and more colonic disease in the other. The parallel results would suggest that the Hispanic race exhibits more extensive disease. Disease extent was different for African-Americans, as the only study reporting on it showed that most African-Americans had rectal disease. Similar to the Hispanic group, the large SA population showed significantly more pan-colonic disease. Overall, these findings imply a higher rate of colonic disease in Hispanics and SA migrants.

An interesting observation borne out by studies in Leicester, North West London and British Columbia<sup>[18,35,38]</sup> is that the well documented increase in pan-colonic disease did not translate into higher colectomy rate, as would be expected. However, the two Hispanic studies in this review showed more colonic disease, and the one that studied colectomy rate reported a higher colectomy rate for UC as would be expected with a higher prevalence of pan-colonic disease<sup>[24]</sup>.

In addition, we recently published data generated from a national database showing a higher rate of colectomy in a SA cohort in the United Kingdom<sup>[41]</sup>.

In contrast to the UC incidence rate, the incidence of CD seems to be consistently lower in SA than Caucasians. Only one study showed a higher incidence rate<sup>[12]</sup>. This was a paediatric population, where the

incidence of CD in the background population was much lower (1.0/10000) than in a parallel Canadian study (11.3/100000). It is plausible to speculate that it may reflect earlier disease onset for second generation migrants. The meta-analyses suggested no strong evidence of a difference in the incidence of the pooled CD data. Were the outlier study by Pinski *et al*<sup>[12]</sup> to be excluded, there would be a consistently lower incidence of CD in SAs. The lower prevalence in the United Kingdom is consistent with the lower CD incidence for SA. In the United States, prevalence for Asians was lower but this group is broader and includes South East Asians and Pacific Asians.

The phenotypic pattern shows more colonic, less ileal disease, more perianal and more penetrating and stricturing disease in SA, with evidence collated from different studies. The two independent observations of more colonic disease and perianal disease in SA are supportive of more aggressive disease in this group. Similarly, the African-Americans had significantly less TI disease, inferring more colonic disease. There was more perianal disease, and even though this stems from only two studies, they represent 65% of the studied cases. The Hispanics also show more colonic and perianal disease. Overall, migrants seem to be less likely to have CD, but when it presents it tends to be more aggressive with colonic and perianal location and complex behaviour.

The prevalence studies show UC and CD to be more prevalent in Caucasians compared to Black and Hispanic groups and UC is more common in SA groups. Comparing the two CD United States studies<sup>[19,20]</sup>, a

significant increase in prevalence was shown over time in all groups. There has only been one United Kingdom prevalence study, which was performed in 1989, and the last United States study was over 10 years ago. Newer studies are needed to update the emergence of disease.

Differences in phenotype were noted between first and second generation SA migrants. In second generation migrants in Leicester, extensive colitis was more common than in the first generation, which was similar to that seen in Caucasians<sup>[15]</sup>. The disease pattern followed that of the indigenous population after only one generation and conveys the influence of environmental factors on disease expression. However, these findings were not replicated in Northwest London less than a decade later. UC disease phenotype in first generation SA diagnosed within 10 years of migration was similar to second generation SA, even though the dominant phenotype was extensive colitis in both generations<sup>[35]</sup>. When the authors performed a subgroup analysis on first generation patients, the rates of diagnosis in the first, second and third decades after migration were comparable and, somewhat unexpectedly, there was no significant increase in disease diagnosis with time spent in the United Kingdom. This result, in conjunction with similar disease phenotype, led the authors to speculate that perhaps genetic susceptibility had a more important role to play. Indeed, novel UC risk alleles have been identified specifically in the North Indian population<sup>[42]</sup>. A recent genome-wide specific study has shown African-specific loci for UC<sup>[43]</sup>, demonstrating the importance of studying non-European populations to better understand the disease.

### Limitations of the study

The quality assessment of the incidence and prevalence studies revealed significant weaknesses in the body of evidence. Almost all the studies are retrospective and, therefore, subject to case ascertainment bias. Only six of thirteen studies used recognised diagnostic criteria, and the majority of the studies were hospital- rather than population-based and relied on ethnicity reporting through medical records or surname recognition. There is a risk of information bias as data may have been collected by different people, especially in the studies spanning a longer time period. Differential loss of follow up is also another source of bias.

Standard disease classification systems, such as Montreal classification, were not used in all studies. Moreover, the prevailing studies have failed to address several confounding variables-medication, smoking, diet, patient choice and clinical decision-making, all of which could affect disease phenotype-and other markers of severe disease, such as requirement for surgery. Moshkovska *et al*<sup>[44]</sup> demonstrated that SA patients had significantly higher concerns with 5-ASA treatments than non-Asian patients, and SA ethnicity

was independently associated with non-adherence, which is relevant as differential exposure to 5-ASA may contribute to more extensive disease. Diagnostic delay increases the risk of Crohn's-related surgery<sup>[27]</sup>, and if access to medical care is limited for certain ethnic groups, it may explain a more aggressive phenotype. Smoking habits differ between ethnic groups. For instance, in the United Kingdom, an estimated 40% of Bangladeshi men regularly smoke compared to the national average of 24%<sup>[45]</sup>. This would be expected to increase susceptibility to Crohn's rather than UC in this group and would may explain the predominant perianal phenotype observed in Bangladeshis in East London<sup>[36]</sup>.

The other limitation rests with terminology and terms used within each study, depending on the population studied. 'Hispanic' is a broad term that can include people from Mexico, Puerto Rico or Cuba. This is an example of a social construct of Hispanic ethnicity, which may not reflect the genetic background of the population. Better categorisation of these groups, according to country of origin, is required to determine whether there are differences. In the African-Americans, the distinction between whether the patients were migrants or second generation was not clear and as we have seen in the SA group, this can impact on disease phenotype. Apart from the SA group, the phenotypic studies do not show clear differences in comparison to Caucasians. Within the Hispanic and African-American groups, the phenotypic results are discordant, which may reflect the heterogeneity of the population.

This review, with its attendant limitations, demonstrated the absence of high-quality, prospective, population-based epidemiological studies on this topic. The ACCESS<sup>[4]</sup> and Epicom<sup>[46]</sup> studies are exemplary recent prospective multicentre cohorts studies that generated new insights into the incidence of IBD in Asia-Pacific and Europe; however, they did not report on migrants. We, subsequently, conducted a retrospective review of the Epicom study population, whereby migrant status was re-examined. We noted a higher than expected incidence of IBD in the migrant population, even though the number of migrants in the majority of countries was still low<sup>[47]</sup>. Conversely, a recent paper describing the study of movement of migrants from high to low incidence areas (Faroe Islands to Denmark) has shown a significant impact on UC disease risk. Excess risk of UC was nearly doubled during the immigrants' first 10 years, but after 10 years the immigrants' UC risk was similar to that of Danes. In this study, the removal of an unknown exposure has caused a dramatic shift in the incidence, emphasising the importance of environmental factors<sup>[48]</sup>.

The key change with migration is exposure to a different environment. This may be driven by factors that change with urbanisation, namely diet, lifestyle (smoking, alcohol) and hygiene, among others. The

diet and its change with migration deserves further examination. Where migrants form an ethnic enclave, it may remain unaltered. When they integrate with the local population, they may adopt the prevailing western diet. Exposure to western diet can be studied by its migration to the east. This tends to be associated with rapid urbanisation, as seen in areas such as Guangzhou, China, where the incidence of IBD has recently risen<sup>[4]</sup>.

Mounting evidence from animal models and human studies indicates an overarching effect of diet on the gut microbiota. Agus *et al.*<sup>[49]</sup> showed how a western diet induces changes in gut microbiota composition, alters host homeostasis and promotes adherent invasive gut colonisation in genetically-susceptible mice. In healthy volunteers, 2 wk of either exclusive animal or plant product consumption altered the microbial community structure and overcame inter-individual differences in microbial gene expression<sup>[50]</sup>. In migrant populations, dietary change may exert a more profound aetio-pathogenic effect, which is largely unexplored in this population group in conjunction with a dearth of detailed dietary data on ethnic groups with IBD. Accurate dietary data is difficult to obtain without an interviewer-led, time-consuming questionnaire. Large scale epidemiological studies often neglect this area in favour of collecting other and easily accessible information.

Studying the diet in migrant populations may provide clues to modifiable risk factors in the development of IBD. Whilst this may appear to be straightforward, in reality it is challenging to explore because patients significantly change their diets at diagnosis, with consequential effects on microbial composition<sup>[51]</sup>. This confounder needs to be recognised and minimised when studying ethnicity and alterations in the microbiota by recording supervised dietary intake. Other environmental factors, such as urbanisation and hygiene, may also influence the microbiome but studying how they do so is more challenging. We suggest looking at the endpoint of genetic and environmental factors by examining the function of microbiota through metabolic profiles in migrant patients.

A link between distinct microbial patterns and migration may partly explain the differences in disease phenotype described in this review. Two studies reported different microbial profiles for ethnic groups<sup>[52,53]</sup>. A further study demonstrated that these compositional differences can drive metabolic and immune activities, which can be related to disease severity<sup>[54]</sup>. Confounders such as smoking, diet, medications, disease phenotype and severity that affect the microbiota were not analysed separately within these studies, due to the small numbers of patients.

The potential role of environmental factors on microbial colonisation is an area of increasing interest<sup>[55]</sup>. Studying these early life perturbations in a genetically predisposed population, such as second generation migrants, bought up in a 'western' environment, for

example SA, may help to decipher the impact of genetic and environmental factors. A prospective inception cohort to describe real-time epidemiological features of IBD will address the limitations observed in this systematic review. Parallel in-depth analysis of microbial factors may show a specific microbial profile related to migration and explain the differing disease phenotype, whilst addressing the confounding factors.

## ARTICLE HIGHLIGHTS

### Research background

Epidemiologic studies suggest an increasing incidence and prevalence of inflammatory bowel disease (IBD) in developed countries. Some studies have reported a change in migrants moving from developing low incidence countries to developed high incidence countries, whereby they exhibit the incidence of the adopted country. This implies there may be an environmental trigger for the disease, as the onset is too rapid to be accounted for by genetic changes.

### Research motivation

Disease presentation following migration offers a unique opportunity to examine how environmental factors might influence disease expression in migrants. Describing epidemiological changes in migrant groups may identify susceptible ethnic groups and help target further studies.

### Research objectives

The authors sought to summarise the current literature on IBD manifestation after migration to developed countries. As there may be an overlap between race, ethnicity and migration, we aimed to study epidemiology of IBD in ethnic migrant and racial groups compared with the indigenous population.

### Research methods

A systematic review using PRISMA guidelines was undertaken. Studies on incidence, prevalence and disease phenotype of migrants and race compared with indigenous groups were eligible for inclusion. A statistical meta-analysis comparing the incidence between migrants and Caucasian groups was performed.

### Research results

Thirty-three studies met the inclusion criteria. South Asians showed consistently higher incidence than indigenous groups for ulcerative colitis (UC). Pooled analysis could only be undertaken for incidence studies on South Asians compared to Caucasians. There was significant heterogeneity between the studies [95% for UC, 83% for Crohn's disease (CD)]. The difference between incidence rates was not significant, with a rate ratio for South Asian:Caucasian of 0.78 (95%CI: 0.22-2.78) for CD and 1.39 (95%CI: 0.84-2.32) for UC. In six studies, there were more South Asians with pan-colonic disease than Caucasians, and this was a significant finding in four studies. A similar pattern was observed for Hispanics in the United States. Bangladeshi and African-Americans showed an increased risk of CD with perianal disease.

### Research conclusions

This the first study to show consistent differences in disease incidence, prevalence and phenotypes between migrants and non-migrants. The South Asian migrant population are particularly susceptible to developing UC. This review has demonstrated the absence of high-quality, prospective, population-based epidemiological studies on this topic. Investigation of migrant populations may provide insight into the role of environmental change and diet in the aetiology of IBD.

### Research perspectives

A prospective inception cohort to describe real-time epidemiological features of IBD will address the limitations observed in this systematic review. The potential role of environmental factors on microbial colonisation is an area of



increasing interest. Studying second generation migrants brought up in a 'western' environment, for example South Asians, may help to decipher the impact of genetic and environmental factors. Parallel in-depth analysis of microbial factors may show a specific microbial profile related to migration and explain differing disease phenotype, whilst addressing confounding factors.

## ACKNOWLEDGMENTS

We would like to thank Paul Bassett for providing statistical support.

## REFERENCES

- 1 **Khor B**, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 2011; **474**: 307-317 [PMID: 21677747 DOI: 10.1038/nature10209]
- 2 **Bernstein CN**, Blanchard JF, Rawsthorne P, Wajda A. Epidemiology of Crohn's disease and ulcerative colitis in a central Canadian province: a population-based study. *Am J Epidemiol* 1999; **149**: 916-924 [PMID: 10342800 DOI: 10.1093/oxfordjournals.aje.a009735]
- 3 **Molodecky NA**, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012; **142**: 46-54.e42; quiz e30 [PMID: 22001864 DOI: 10.1053/j.gastro.2011.10.001]
- 4 **Ng SC**, Tang W, Ching JY, Wong M, Chow CM, Hui AJ, Wong TC, Leung VK, Tsang SW, Yu HH, Li MF, Ng KK, Kamm MA, Studd C, Bell S, Leong R, de Silva HJ, Kasturiratne A, Mufeen MN, Ling KL, Ooi CJ, Tan PS, Ong D, Goh KL, Hilmi I, Pisespongsa P, Manatsathit S, Rerknimitr R, Aniwan S, Wang YF, Ouyang Q, Zeng Z, Zhu Z, Chen MH, Hu PJ, Wu K, Wang X, Simadibrata M, Abdullah M, Wu JC, Sung JJ, Chan FK; Asia-Pacific Crohn's and Colitis Epidemiologic Study (ACCESS) Study Group. Incidence and phenotype of inflammatory bowel disease based on results from the Asia-Pacific Crohn's and colitis epidemiology study. *Gastroenterology* 2013; **145**: 158-165.e2 [PMID: 23583432 DOI: 10.1053/j.gastro.2013.04.007]
- 5 **Probert CS**, Jayanthi V, Pinder D, Wicks AC, Mayberry JF. Epidemiological study of ulcerative proctocolitis in Indian migrants and the indigenous population of Leicestershire. *Gut* 1992; **33**: 687-693 [PMID: 1307684]
- 6 **Probert CS**, Jayanthi V, Pollock DJ, Baithun SI, Mayberry JF, Rampton DS. Crohn's disease in Bangladeshis and Europeans in Britain: an epidemiological comparison in Tower Hamlets. *Postgrad Med J* 1992; **68**: 914-920 [PMID: 1494514 DOI: 10.1136/pgmj.68.805.914]
- 7 **Davis KF**, D'Odorico P, Laio F, Ridolfi L. Global spatio-temporal patterns in human migration: a complex network perspective. *PLoS One* 2013; **8**: e53723 [PMID: 23372664 DOI: 10.1371/journal.pone.0053723]
- 8 **Pais P**, Pogue J, Gerstein H, Zachariah E, Savitha D, Jayprakash S, Nayak PR, Yusuf S. Risk factors for acute myocardial infarction in Indians: a case-control study. *Lancet* 1996; **348**: 358-363 [PMID: 8709733]
- 9 **Fernando E**, Razak F, Lear SA, Anand SS. Cardiovascular Disease in South Asian Migrants. *Can J Cardiol* 2015; **31**: 1139-1150 [PMID: 26321436 DOI: 10.1016/j.cjca.2015.06.008]
- 10 **Bhopal RS**. Ethnicity, race, and health in multicultural societies. 1<sup>st</sup> edit. Oxford University Press (Great Clarendon Street, Oxford OX2 6DP, United Kingdom); 2007
- 11 **Fellows IW**, Freeman JG, Holmes GK. Crohn's disease in the city of Derby, 1951-1985. *Gut* 1990; **31**: 1262-1265 [PMID: 2253910 DOI: 10.1136/gut.31.11.1262]
- 12 **Pinsk V**, Lemberg DA, Grewal K, Barker CC, Schreiber RA, Jacobson K. Inflammatory bowel disease in the South Asian pediatric population of British Columbia. *Am J Gastroenterol* 2007; **102**: 1077-1083 [PMID: 17378907 DOI: 10.1111/j.1572-0241.2007.01124.x]
- 13 **Benchimol EI**, Mack DR, Guttman A, Nguyen GC, To T, Mojaverian N, Quach P, Manuel DG. Inflammatory bowel disease in immigrants to Canada and their children: a population-based cohort study. *Am J Gastroenterol* 2015; **110**: 553-563 [PMID: 25756238 DOI: 10.1038/ajg.2015.52]
- 14 **Jayanthi V**, Probert CS, Pollock DJ, Baithun SI, Rampton DS, Mayberry JF. Low incidence of ulcerative colitis and proctitis in Bangladeshi migrants in Britain. *Digestion* 1992; **52**: 34-42 [PMID: 1426695]
- 15 **Carr I**, Mayberry JF. The effects of migration on ulcerative colitis: a three-year prospective study among Europeans and first- and second- generation South Asians in Leicester (1991-1994). *Am J Gastroenterol* 1999; **94**: 2918-2922 [PMID: 10520845 DOI: 10.1016/S0002-9270(99)00494-3]
- 16 **Probert CS**, Jayanthi V, Mayberry JF. Inflammatory bowel disease in Indian migrants in Fiji. *Digestion* 1991; **50**: 82-84 [PMID: 1804736]
- 17 **Jayanthi V**, Probert CS, Pinder D, Wicks AC, Mayberry JF. Epidemiology of Crohn's disease in Indian migrants and the indigenous population in Leicestershire. *Q J Med* 1992; **82**: 125-138 [PMID: 1620813 DOI: 10.1093/oxfordjournals.qjmed.a068653]
- 18 **Probert CS**, Jayanthi V, Hughes AO, Thompson JR, Wicks AC, Mayberry JF. Prevalence and family risk of ulcerative colitis and Crohn's disease: an epidemiological study among Europeans and south Asians in Leicestershire. *Gut* 1993; **34**: 1547-1551 [PMID: 8244142 DOI: 10.1136/gut.34.11.1547]
- 19 **Kurata JH**, Kantor-Fish S, Frankl H, Godby P, Vadheim CM. Crohn's disease among ethnic groups in a large health maintenance organization. *Gastroenterology* 1992; **102**: 1940-1948 [PMID: 1587413]
- 20 **Wang YR**, Loftus EV Jr, Cangemi JR, Picco MF. Racial/Ethnic and regional differences in the prevalence of inflammatory bowel disease in the United States. *Digestion* 2013; **88**: 20-25 [PMID: 23797316 DOI: 10.1159/000350759]
- 21 **Lee YM**, Fock K, See SJ, Ng TM, Khor C, Teo EK. Racial differences in the prevalence of ulcerative colitis and Crohn's disease in Singapore. *J Gastroenterol Hepatol* 2000; **15**: 622-625 [PMID: 10921415 DOI: 10.1046/j.1440-1746.2000.02212.x]
- 22 **Li BH**, Guan X, Vittinghoff E, Gupta N. Comparison of the presentation and course of pediatric inflammatory bowel disease in South Asians with Whites: a single center study in the United States. *J Pediatr* 2013; **163**: 1211-1213 [PMID: 23706360 DOI: 10.1016/j.jpeds.2013.04.017]
- 23 **Cross RK**, Jung C, Wasan S, Joshi G, Sawyer R, Roghmann MC. Racial differences in disease phenotypes in patients with Crohn's disease. *Inflamm Bowel Dis* 2006; **12**: 192-198 [PMID: 16534420 DOI: 10.1097/01.MIB.0000217767.98389.20]
- 24 **Nguyen GC**, Torres EA, Regueiro M, Bromfield G, Bitton A, Stempak J, Dassopoulos T, Schumm P, Gregory FJ, Griffiths AM, Hanauer SB, Hanson J, Harris ML, Kane SV, Orkwis HK, Lahaie R, Oliva-Hemker M, Pare P, Wild GE, Rioux JD, Yang H, Duerr RH, Cho JH, Steinhardt AH, Brant SR, Silverberg MS. Inflammatory bowel disease characteristics among African Americans, Hispanics, and non-Hispanic Whites: characterization of a large North American cohort. *Am J Gastroenterol* 2006; **101**: 1012-1023 [PMID: 16696785 DOI: 10.1111/j.1572-0241.2006.00504.x]
- 25 **Eidelwein AP**, Thompson R, Fiorino K, Abadom V, Oliva-Hemker M. Disease presentation and clinical course in black and white children with inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2007; **44**: 555-560 [PMID: 17460486]
- 26 **Hattar LN**, Abraham BP, Malaty HM, Smith EO, Ferry GD. Inflammatory bowel disease characteristics in Hispanic children in Texas. *Inflamm Bowel Dis* 2012; **18**: 546-554 [PMID: 21456045 DOI: 10.1002/ibd.21698]
- 27 **Ghazi LJ**, Lydecker AD, Patil SA, Rustgi A, Cross RK, Flasar MH. Racial differences in disease activity and quality of life in patients with Crohn's disease. *Dig Dis Sci* 2014; **59**: 2508-2513 [PMID: 24718861 DOI: 10.1007/s10620-014-3141-3]

- 28 **Damas OM**, Jahann DA, Reznik R, McCauley JL, Tamariz L, Deshpande AR, Abreu MT, Sussman DA. Phenotypic manifestations of inflammatory bowel disease differ between Hispanics and non-Hispanic whites: results of a large cohort study. *Am J Gastroenterol* 2013; **108**: 231-239 [PMID: 23247580 DOI: 10.1038/ajg.2012.393]
- 29 **Kugathasan S**, Judd RH, Hoffmann RG, Heikenen J, Telega G, Khan F, Weisdorf-Schindele S, San Pablo W Jr, Perrault J, Park R, Yaffe M, Brown C, Rivera-Bennett MT, Halabi I, Martinez A, Blank E, Werlin SL, Rudolph CD, Binion DG; Wisconsin Pediatric Inflammatory Bowel Disease Alliance. Epidemiologic and clinical characteristics of children with newly diagnosed inflammatory bowel disease in Wisconsin: a statewide population-based study. *J Pediatr* 2003; **143**: 525-531 [PMID: 14571234 DOI: 10.1067/S0022-3476(03)00444-X]
- 30 **Malaty HM**, Hou JK, Thirumurthi S. Epidemiology of inflammatory bowel disease among an indigent multi-ethnic population in the United States. *Clin Exp Gastroenterol* 2010; **3**: 165-170 [PMID: 21694862 DOI: 10.2147/CEG.S14586]
- 31 **Adler J**, Dong S, Eder SJ, Dombkowski KJ; ImproveCareNow Pediatric IBD Learning Health System. Perianal Crohn Disease in a Large Multicenter Pediatric Collaborative. *J Pediatr Gastroenterol Nutr* 2017; **64**: e117-e124 [PMID: 27801750]
- 32 **Basu D**, Lopez I, Kulkarni A, Sellin JH. Impact of race and ethnicity on inflammatory bowel disease. *Am J Gastroenterol* 2005; **100**: 2254-2261 [PMID: 16181378 DOI: 10.1111/j.1572-0241.2005.00233.x]
- 33 **Moore L**, Gaffney K, Lopez R, Shen B. Comparison of the natural history of ulcerative colitis in African Americans and non-Hispanic Caucasians: a historical cohort study. *Inflamm Bowel Dis* 2012; **18**: 743-749 [PMID: 21688351 DOI: 10.1002/ibd.21796]
- 34 **Flasar MH**, Quezada S, Bijpuria P, Cross RK. Racial differences in disease extent and severity in patients with ulcerative colitis: a retrospective cohort study. *Dig Dis Sci* 2008; **53**: 2754-2760 [PMID: 18273704 DOI: 10.1007/s10620-007-0190-x]
- 35 **Walker DG**, Williams HR, Kane SP, Mawdsley JE, Arnold J, McNeil I, Thomas HJ, Teare JP, Hart AL, Pitcher MC, Walters JR, Marshall SE, Orchard TR. Differences in inflammatory bowel disease phenotype between South Asians and Northern Europeans living in North West London, UK. *Am J Gastroenterol* 2011; **106**: 1281-1289 [PMID: 21577243 DOI: 10.1038/ajg.2011.85]
- 36 **Goodhand JR**, Kamperidis N, Joshi NM, Wahed M, Koodun Y, Cantor EJ, Croft NM, Langmead FL, Lindsay JO, Rampton DS. The phenotype and course of inflammatory bowel disease in UK patients of Bangladeshi descent. *Aliment Pharmacol Ther* 2012; **35**: 929-940 [PMID: 22404452 DOI: 10.1111/j.1365-2036.2012.05043.x]
- 37 **Rashid ST**, Bharucha S, Jamallulail SI, Banait GS, Kemp K, Makin A, Newman WG. Inflammatory bowel disease in the South Asian population of Northwest England. *Am J Gastroenterol* 2008; **103**: 242-243; author reply 243-244 [PMID: 18184129 DOI: 10.1111/j.1572-0241.2007.01562\_3.x]
- 38 **Carroll MW**, Hamilton Z, Gill H, Simkin J, Smyth M, Espinosa V, Bressler B, Jacobson K. Pediatric Inflammatory Bowel Disease Among South Asians Living in British Columbia, Canada: A Distinct Clinical Phenotype. *Inflamm Bowel Dis* 2016; **22**: 387-396 [PMID: 26752467 DOI: 10.1097/MIB.0000000000000651]
- 39 **Hilmi I**, Singh R, Ganesanathan S, Yatim I, Radzi M, Chua AB, Tan HJ, Huang S, Chin KS, Menon J, Goh KL. Demography and clinical course of ulcerative colitis in a multiracial Asian population: a nationwide study from Malaysia. *J Dig Dis* 2009; **10**: 15-20 [PMID: 19236542 DOI: 10.1111/j.1751-2980.2008.00357.x]
- 40 **Sofia MA**, Rubin DT, Hou N, Pekow J. Clinical presentation and disease course of inflammatory bowel disease differs by race in a large tertiary care hospital. *Dig Dis Sci* 2014; **59**: 2228-2235 [PMID: 24752402 DOI: 10.1007/s10620-014-3160-0]
- 41 **Misra R**, Askari A, Faiz O, Arebi N. Colectomy Rates for Ulcerative Colitis Differ between Ethnic Groups: Results from a 15-Year Nationwide Cohort Study. *Can J Gastroenterol Hepatol* 2016; **2016**: 8723949 [PMID: 28074174 DOI: 10.1155/2016/8723949]
- 42 **Juyal G**, Negi S, Sood A, Gupta A, Prasad P, Senapati S, Zaneveld J, Singh S, Midha V, van Sommeren S, Weersma RK, Ott J, Jain S, Juyal RC, Thelma BK. Genome-wide association scan in north Indians reveals three novel HLA-independent risk loci for ulcerative colitis. *Gut* 2015; **64**: 571-579 [PMID: 24837172 DOI: 10.1136/gutjnl-2013-306625]
- 43 **Brant SR**, Okou DT, Simpson CL, Cutler DJ, Haritunians T, Bradfield JP, Chopra P, Prince J, Begum F, Kumar A, Huang C, Venkateswaran S, Datta LW, Wei Z, Thomas K, Herrinton LJ, Klapproth JA, Quiros AJ, Seminerio J, Liu Z, Alexander JS, Baldassano RN, Dudley-Brown S, Cross RK, Dassopoulos T, Denson LA, Dhere TA, Dryden GW, Hanson JS, Hou JK, Hussain SZ, Hyams JS, Isaacs KL, Kader H, Kappelman MD, Katz J, Kellermayer R, Kirschner BS, Kuemmerle JF, Kwon JH, Lazarev M, Li E, Mack D, Mannon P, Moulton DE, Newberry RD, Osuntokun BO, Patel AS, Saeed SA, Targan SR, Valentine JF, Wang MH, Zonca M, Rioux JD, Duerr RH, Silverberg MS, Cho JH, Hakonarson H, Zwick ME, McGovern DP, Kugathasan S. Genome-Wide Association Study Identifies African-Specific Susceptibility Loci in African Americans With Inflammatory Bowel Disease. *Gastroenterology* 2017; **152**: 206-217.e2 [PMID: 27693347 DOI: 10.1053/j.gastro.2016.09.032]
- 44 **Moshkovska T**, Stone MA, Clatworthy J, Smith RM, Bankart J, Baker R, Wang J, Horne R, Mayberry JF. An investigation of medication adherence to 5-aminosalicylic acid therapy in patients with ulcerative colitis, using self-report and urinary drug excretion measurements. *Aliment Pharmacol Ther* 2009; **30**: 1118-1127 [PMID: 19785623 DOI: 10.1111/j.1365-2036.2009.04152.x]
- 45 **Sprotson K**, Mindell J. The health of minority ethnic groups - Summary of key findings. 2004. Available from: URL: <http://digital.nhs.uk/catalogue/PUB01170>
- 46 **Burisch J**, Pedersen N, Čuković-Čavka S, Brinar M, Kaimakliotis I, Duricova D, Shonová O, Vind I, Avnstrøm S, Thorsgaard N, Andersen V, Krabbe S, Dahlerup JF, Salupere R, Nielsen KR, Olsen J, Manninen P, Collin P, Tsianos EV, Katsanos KH, Ladefoged K, Lakatos L, Björnsson E, Ragnarsson G, Bailey Y, Odes S, Schwartz D, Martinato M, Lupinacci G, Milla M, De Padova A, D'Inca R, Beltrami M, Kupcinskis L, Kiudelis G, Turcan S, Tighineanu O, Mihiu I, Magro F, Barros LF, Goldis A, Lazar D, Belousova E, Nikulina I, Hernandez V, Martinez-Ares D, Almer S, Zhulina Y, Halfvarson J, Arebi N, Sebastian S, Lakatos PL, Langholz E, Munkholm P; EpiCom-group. East-West gradient in the incidence of inflammatory bowel disease in Europe: the ECCO-EpiCom inception cohort. *Gut* 2014; **63**: 588-597 [PMID: 23604131 DOI: 10.1136/gutjnl-2013-304636]
- 47 **Misra R**, Burisch J, Haji S, Salupere R, Ellul P, Ramirez V, D'Inca R, Munkholm PAN. Impact of migration on IBD incidence in 8 European populations: results from Epicom 2010 inception cohort study. In: European Crohn's and Colitis Organisation. Barcelona: 2017
- 48 **Hammer T**, Lophaven SN, Nielsen KR, von Euler-Chelpin M, Weihe P, Munkholm P, Burisch J, Lyng E. Inflammatory bowel diseases in Faroese-born Danish residents and their offspring: further evidence of the dominant role of environmental factors in IBD development. *Aliment Pharmacol Ther* 2017; **45**: 1107-1114 [PMID: 28176348]
- 49 **Agus A**, Denizot J, Thévenot J, Martinez-Medina M, Massier S, Sauvanet P, Bernalier-Donadille A, Denis S, Hofman P, Bonnet R, Billard E, Barnich N. Western diet induces a shift in microbiota composition enhancing susceptibility to Adherent-Invasive *E. coli* infection and intestinal inflammation. *Sci Rep* 2016; **6**: 19032 [PMID: 26742586 DOI: 10.1038/srep19032]
- 50 **David LA**, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 2014; **505**: 559-563 [PMID: 24336217 DOI: 10.1038/nature12820]
- 51 **Limdi JK**, Aggarwal D, McLaughlin JT. Dietary Practices and Beliefs in Patients with Inflammatory Bowel Disease. *Inflamm Bowel Dis* 2016; **22**: 164-170 [PMID: 26383912 DOI: 10.1097/

- MIB.0000000000000585]
- 52 **Prideaux L**, Kang S, Wagner J, Buckley M, Mahar JE, De Cruz P, Wen Z, Chen L, Xia B, van Langenberg DR, Lockett T, Ng SC, Sung JJ, Desmond P, McSweeney C, Morrison M, Kirkwood CD, Kamm MA. Impact of ethnicity, geography, and disease on the microbiota in health and inflammatory bowel disease. *Inflamm Bowel Dis* 2013; **19**: 2906-2918 [PMID: 24240708 DOI: 10.1097/01.MIB.0000435759.05577.12]
  - 53 **Rehman A**, Rausch P, Wang J, Skieceviciene J, Kiudelis G, Bhagalia K, Amarapurkar D, Kupcinskas L, Schreiber S, Rosenstiel P, Baines JF, Ott S. Geographical patterns of the standing and active human gut microbiome in health and IBD. *Gut* 2016; **65**: 238-248 [PMID: 25567118 DOI: 10.1136/gutjnl-2014-308341]
  - 54 **Mar JS**, LaMere BJ, Lin DL, Levan S, Nazareth M, Mahadevan U, Lynch S V. Disease Severity and Immune Activity Relate to Distinct Interkingdom Gut Microbiome States in Ethnically Distinct Ulcerative Colitis Patients. *MBio* 2016; **7**: e01072-16 [PMID: 27531910 DOI: 10.1128/mBio.01072-16]
  - 55 **Gensollen T**, Iyer SS, Kasper DL, Blumberg RS. How colonization by microbiota in early life shapes the immune system. *Science* 2016; **352**: 539-544 [PMID: 27126036 DOI: 10.1126/science.aad9378]

**P- Reviewer:** Krishnan T, Specchia ML **S- Editor:** Ma YJ  
**L- Editor:** Filipodia **E- Editor:** Huang Y



## Beneficial long term effect of a phosphodiesterase-5-inhibitor in cirrhotic portal hypertension: A case report with 8 years follow-up

Peter Deibert, Adhara Lazaro, Zoran Stankovic, Denise Schaffner, Martin Rössle, Wolfgang Kreisel

Peter Deibert, Adhara Lazaro, Denise Schaffner, Faculty of Medicine, Institute for Exercise and Occupational Medicine, Department of Medicine, University Hospital, Freiburg 79106, Germany

Zoran Stankovic, Inselspital, Interventional and Pediatric Radiology, Institute of Diagnostic, University of Bern, Bern 3010, Switzerland

Denise Schaffner, Department of Pharmaceutical Biology and Biotechnology, University of Freiburg, Freiburg 79106, Germany

Martin Rössle, Private Praxis, Praxiszentrum, Freiburg 79104, Germany

Wolfgang Kreisel, Faculty of Medicine, Endocrinology and Infectious Diseases, Department of Gastroenterology, Hepatology, University Hospital, Freiburg 79106, Germany

ORCID number: Peter Deibert (0000-0002-3291-884X); Adhara Lazaro (0000-0001-9023-1387); Zoran Stankovic (0000-0001-6265-3072); Denise Schaffner (0000-0001-6173-9288); Martin Rössle (0000-0002-1358-9163); Wolfgang Kreisel (0000-0001-6884-0135).

**Author contributions:** Deibert P and Kreisel W treated the patient and designed the concept of treatment of portal hypertension with PDE-5-inhibitors; Stankovic Z performed the MRI measurements; Deibert P did the sonographic examinations; Rössle M, Kreisel W and Deibert P performed the invasive measurements of HVP; All authors shared the acquired data; Deibert P, Lazaro A, Schaffner D, Rössle M and Kreisel W drafted the manuscript.

**Informed consent statement:** The described patient provided informed written consent prior to study enrollment.

**Conflict-of-interest statement:** None of the authors declared a conflict of interest.

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**Manuscript source:** Unsolicited manuscript

**Correspondence to:** Wolfgang Kreisel, MD, Professor, Department of Internal Medicine, University of Freiburg, Hugstetter Str. 55, Freiburg 79106, Germany. [wolfgang.kreisel@uniklinik-freiburg.de](mailto:wolfgang.kreisel@uniklinik-freiburg.de)  
Telephone: +49-761-27034010  
Fax: +49-761-27074880

**Received:** October 27, 2017

**Peer-review started:** October 28, 2017

**First decision:** November 14, 2017

**Revised:** December 1, 2017

**Accepted:** December 4, 2017

**Article in press:** December 4, 2017

**Published online:** January 21, 2018

### Abstract

Non-selective beta-blockers are the mainstay of medical therapy for portal hypertension in liver cirrhosis. Inhibitors of phosphodiesterase-5 (PDE-5-inhibitors) reduce portal pressure in the acute setting by > 10% which may suggest a long-term beneficial effect. Currently, there is no available data on long-term treatment of portal hypertension with PDE-5-inhibitors. This case of a patient with liver cirrhosis secondary to autoimmune liver disease with episodes of bleeding from esophageal varices is the first documented case in which a treatment with a PDE-5-inhibitor for eight years was monitored. In the acute setting, the PDE-5-inhibitor Vardenafil lowered portal pressure by 13%. The portal blood flow increased by 28% based on



Doppler sonography and by 16% using MRI technique. As maintenance medication the PDE-5-inhibitor Tadalafil was used for eight consecutive years with comparable effects on portal pressure and portal blood flow. There were no recurrence of bleeding and no formation of new varices. Influencing the NO-pathway by the use of PDE-5 inhibitors may have long-term beneficial effects in compensated cirrhosis.

**Key words:** Portal hypertension; Phosphodiesterase-5; Liver hemodynamics; Doppler sonography; Magnetic resonance imaging; Liver cirrhosis

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**Core tip:** Non-selective beta-blockers are the mainstay of medical therapy for portal hypertension in liver cirrhosis. Inhibitors of phosphodiesterase-5 (PDE-5) reduce portal pressure in the acute setting by > 10%. This is the first report of a patient with liver cirrhosis showing that in long-term treatment with a PDE-5-inhibitor the positive effect on liver hemodynamics is maintained thus preventing further variceal bleeding.

Deibert P, Lazaro A, Stankovic Z, Schaffner D, Rössle M, Kreisel W. Beneficial long term effect of a phosphodiesterase-5-inhibitor in cirrhotic portal hypertension: A case report with 8 years follow-up. *World J Gastroenterol* 2018; 24(3): 438-444 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i3/438.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i3.438>

## INTRODUCTION

Portal hypertension in liver cirrhosis is caused by several factors<sup>[1-3]</sup>. Structural changes (*i.e.*, regenerative nodules and fibrosis) lead to an intrahepatic outflow obstruction which is aggravated by an increased portal blood flow (at least in early stages of cirrhosis) as a consequence of excessive extrahepatic nitric oxide (NO) production. About a quarter of the portal pressure is due to a functional intrahepatic component: the imbalance between constricting and dilating factors within the sinusoids and the dysregulation of the nitric oxide - cyclic guanosine monophosphate (NO-cGMP) system lead to an overactivity of stellate cells/myofibroblasts and contraction of sinusoids. Phosphodiesterase-5-inhibitors (PDE-5-inhibitors) inhibit the conversion of cGMP to 5'-GMP<sup>[2]</sup> thus, increasing the level of cGMP, the second messenger, which may lead to dilation of sinusoids. There are conflicting data from preclinical and clinical studies whether or not PDE-5-Inhibitors lower elevated portal pressure in cirrhosis<sup>[4-6]</sup>. However, a recent proof-of-concept study showed that the long-acting PDE-5-inhibitor Udenafil lowered portal pressure at doses of 75-100 mg by approximately 20% in the acute testing<sup>[7]</sup>.

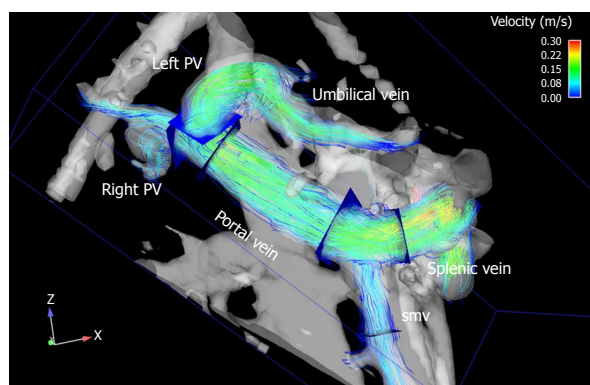
A decrease of hepatovenous pressure gradient (HVPG) by  $\geq 20\%$  from baseline or to a value of  $\leq 12$  mm Hg after an application of non-selective beta-blockers for more than two weeks is accepted as a good predictor of beneficial clinical response<sup>[8-10]</sup>. Meanwhile, several studies demonstrated that a decrease of HVPG by  $\geq 10\%$  in the acute setting after intravenous administration of propranolol (0.15 mg/kg) is an adequate predictor of a clinical response as well<sup>[11-13]</sup>. Therefore, it may be surmised that a decrease of HVPG by  $\geq 10\%$  in the acute setting after application of a PDE-5-inhibitor may be an indicator of a long-term beneficial effect in liver cirrhosis with portal hypertension.

In this case report of a patient with liver cirrhosis who had multiple episodes of bleeding esophageal varices we aimed to answer the following questions: (1) Are the decrease of portal pressure and increase of portal venous blood flow induced by a PDE-5-inhibitor long-lasting? (2) Does the long-term use of a PDE-5-inhibitor have a beneficial clinical effect of reducing the risk of bleeding from esophageal varices? And (3) Do we see any compound-specific adverse effects in the long-term?

## CASE REPORT

In February 2009 a 53-year-old female patient was admitted to the University Hospital in Freiburg, Germany for further evaluation. The patient was initially diagnosed with autoimmune hepatitis/primary biliary cholangitis (AIH/PBC) overlap syndrome (positive for anti-nuclear antibodies, antibodies to smooth muscle cells, antibodies to pyruvate decarboxylase E2 subunit, and to soluble liver antigen) in 1989. Since histology and clinical chemistry showed that the AIH component was predominant without cholestasis, the prescribed therapy was a combination of corticosteroids and azathioprine.

In 2006 the patient was previously admitted in the Hospital of Norden, Germany with the first episode of acute variceal bleeding with hemodynamic instability. Banding of varices was performed. Sonography of the abdominal organs showed signs of liver cirrhosis including enlarged spleen. No other abnormalities were found. Within the following 3 years she bled three times per year from these varices. In 2008 she had two episodes of bleeding from rectal varices which were treated with rubber band ligation. Medical therapy of portal hypertension with propranolol was initiated but had to be stopped even at the low dose of 20 mg twice daily due to intolerable cardiovascular side effects (*i.e.*, bradycardia and hypotension). The patient was regularly monitored in the liver transplant center of the University Hospital in Hannover (Medizinische Hochschule Hannover). She was waitlisted for liver transplantation and the implantation of TIPS was scheduled. She was referred to our hepatological unit in



**Figure 1** Time-resolved 3D MRI image shows prograde blood flow in the extrahepatic portal venous system and a recanalization of the umbilical vein with flow over the left branch of the intrahepatic portal vein. PV: Portal vein; smv: Superior mesenteric vein.

order to check whether the application of an inhibitor of the enzyme phosphodiesterase-5 could be an option to lower portal pressure and reduce the risk for bleeding from esophageal varices.

The patient was in a good clinical condition upon admission. The physical examination findings were normal except for the palpable liver and minor bilateral varicose veins of the lower extremities. Examination of heart and lungs was unremarkable. No bipedal edema was present. There were no signs of hepatic encephalopathy. Blood pressure was 154/86 mmHg and the heart rate was 78/min. ECG and echocardiography findings were normal except for a slight tricuspid valve insufficiency. Systolic pulmonary artery pressure was not increased (26 mmHg). Laboratory results showed no pathological values except a slight increase in total bilirubin (1.6 mg/dL) and thrombocytopenia of 105,000/ $\mu$ L, establishing the case as Child A cirrhosis (*i.e.*, normal bilirubin, normal serum albumin, normal INR, no ascites, no hepatic encephalopathy). In esophago-gastro-duodenoscopy scarring transformation of two ligated varices was observed. Three small varices of grade 1-2 were visible. At this point, the daily medication consisted of azathioprine 75 mg, prednisolone 5 mg, pantoprazole 20 mg, and calcium 500 mg.

To test the effect of a PDE-5-inhibitor on portal hemodynamics in February 2009 10 mg of Vardenafil were administered orally. Wedged hepatic vein pressure (WHVP) and free hepatic vein pressure (FHVP) were measured in triplicate before and one hour after the drug administration. The HVPG, defined as WHVP - FHVP, decreased by 14% from 10.5 to 9.0 mmHg. Duplex sonography showed an increase in portal flow by 28% (0.97 L/min to 1.24 L/min) 60 min after drug intake. Systemic blood pressure changed from 130/87 to 121/75 mmHg one hour after drug administration, while heart rate changed from 65 to 61/min. Portal flow monitored by flow-sensitive 3D magnetic resonance imaging increased by 16 % (0.85 L/min to 0.99 L/min).

Maximal flow velocity remained constant, at 24 cm/s in duplex sonography and 17 cm/s in 3D MRI. No relevant effect was observed on systemic blood pressure. Visualization of the portal venous system by MRI (Figure 1) confirmed the duplex sonographic findings of a prograde blood flow in the portal vein and the two main intrahepatic branches and a recanalized umbilical vein originating from the left main branch of the portal vein.

We discussed an experimental therapeutic approach of the application of a PDE-5-inhibitor with the patient. The patient was informed that this was an off-label use and gave written informed consent. After having verified that the PDE-5-inhibitor Vardenafil decreased HVPG and that it led to an increase of portal venous blood flow confirmed by two independent methods we decided to start a long-term therapy with 5 mg Tadalafil/day, as this PDE-5-inhibitor has a longer half-life than Vardenafil. In June 2009 an HVPG one hour after oral administration of 5 mg Tadalafil of 10.5 mmHg was measured. Portal venous blood flow remained elevated at 1.21 L/min. Systemic blood pressure showed no clinically relevant changes (124/79 mmHg, heart rate 88/min to 127/77 mmHg, heart rate 64/min) in the acute setting. The next evaluation was performed in October 2009 wherein after 10 mg Tadalafil the HVPG decreased by 15% from 12.0 to 10.0 mmHg. Portal venous blood flow remained markedly elevated (1.28 L/min in Duplex sonography and 0.99 L/min as measured by MRI). In March 2010 HVPG was 10.5 mmHg, while portal venous blood flow remained about 30% higher than the initial reading as verified by the two methods. Notably, the flow in the umbilical vein was not influenced by the PDE-5-inhibitor. The results of the measurements are shown in Table 1. No relevant changes in heart rate or blood pressure occurred. In April 2010 a second-grade esophageal varix was injected with acryl-glue prophylactically. In March 2011 the last measurement of portal blood flow using duplex sonography and the MRI method was performed. It showed that the portal venous blood flow remained constant at a level above than at the start of treatment. Further invasive portal pressure measurements were not performed.

The clinical course of the patient remained stable. The portal venous blood flow determined by duplex sonography was constant within the range of 1.2 and 1.4 L/min. Endoscopic monitoring every six months showed scarring in the distal esophagus and with no signs of new esophageal varices. The varices remained closed by thrombosis. Two episodes of upper gastrointestinal bleeding occurred in 02/2016 and 04/2016. Upper endoscopy excluded bleeding from varices or portal hypertensive gastropathy. The bleeding from the small visible erosions in the duodenum was attributed to NSAID use which the patient had taken due to headache. After administration of a proton pump inhibitor no further bleeding occurred. In 12/2016 portal flow was quantified to be 1.06 L/min. However,

**Table 1** Measurements of hepatic hemodynamic parameters

Time		Flow portal vein (L/min)		Flow umbi-lical vein (L/min)	HVPG (mmHg)	Blood pressure (mmHg)	Heart rate (beats/min)
		Duplex	MRI				
Start	02/2009	0.97	0.85	0.53	10.5	130/87	69
1 h post Vardenafil 10 mg	02/2009	1.24	0.99	0.56	9.0 (-14%)	121/75	61
4 mo Tadalafil 5 mg/d	06/2009		1.21	0.56	10.5	127/77	64
Without PDE-5-I	10/2009				12		
1h post Tadalafil 5 mg	10/2009	1.28	0.99	0.53	10.0 (-15%)	110/70	84
11 mo Tadalafil 5 mg/d	03/2010	1.32	1.33		10.5	120/80	70
20 mo Tadalafil 5 mg/d	10/2010	1.34				115/75	77
26 mo Tadalafil 5 mg/d	04/2011	1.26	1.34			125/80	72
32 mo Tadalafil 5 mg/d	10/2011	1.25				117/70	66
46 mo Tadalafil 5 mg/d	12/2012	1.20				131/85	70
53 mo Tadalafil 5 mg/d	07/2013	1.20				110/70	68
60 mo Tadalafil 5 mg/d	02/2014	1.20				115/75	78
70 mo Tadalafil 5 mg/d	12/2014	1.20				110/70	70
81 mo Tadalafil 5 mg/d	11/2015	1.40				135/85	65
84 mo Tadalafil 5 mg/d	12/2016	1.06 <sup>1</sup>				135/75	85
93 mo Tadalafil 5 mg/d	09/2017	1.29				110/70	80

<sup>1</sup>The duplexsonography at month 84 was done with a different device.

this was measured with a different sonographic device. The next sonographic examination in 09/2017 with the original device revealed a constant portal blood flow of 1.29 L/min.

Figure 2 shows the distal aspect of the esophagus with visible scars caused by sclerosing and banding, with the old varices remained thrombosed. Figure 3 shows an abdominal computer tomographic angiography. The portal vein (solid arrow) was perfused in a prograde direction, the diameter was enlarged. There were collateral veins at the splenic hilus and an enlarged left ovarian vein (dashed arrows).

The patient continued the daily intake of 5 mg Tadalafil. Other medications consisted of 75 mg Azathioprine per day and 2.5 mg Prednisone per day. Because of the stable clinical condition the patient is no longer on the list of liver transplant candidates. During the time of follow-up the number of varicose veins of the lower extremities increased and a local therapy was suggested.

## DISCUSSION

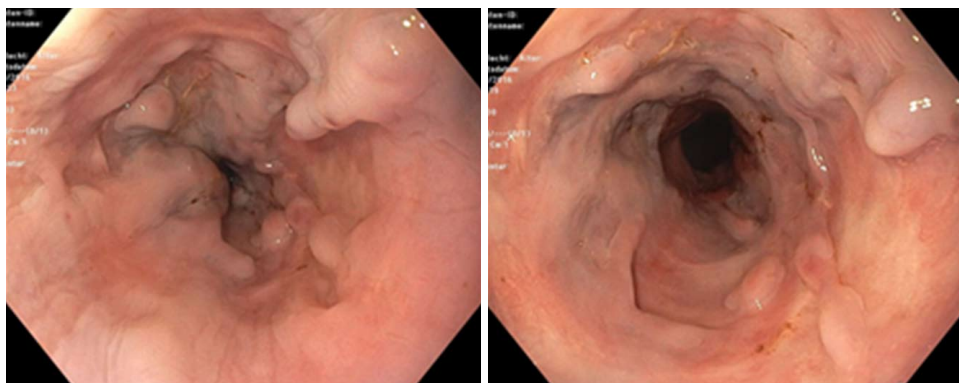
This case report describes a consistent beneficial effect of a PDE-5-inhibitor in a patient with portal hypertension due to AIH/PBC overlap syndrome who had bled from esophageal varices. HVPG decreased by 14% at the initial hemodynamic test and by 15% a few months later. Portal venous flow increased by 28% as measured by Doppler ultrasound and by 16% as measured by four-dimensional flow MRI<sup>[14,15]</sup>. These measurements persisted for more than eight years and were accompanied by a beneficial clinical effect. Since the start of a therapy with 5 mg Tadalafil per day no further esophageal variceal bleeding occurred for eight years. Compound-specific adverse effects were not observed. In particular, no clinically significant side

effects to systemic hemodynamics were detected.

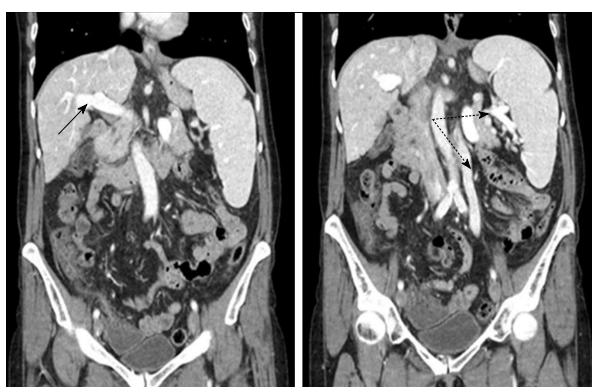
For several years the question whether or not an inhibition of the enzyme PDE-5 lowers portal hypertension in patients with liver cirrhosis remained unanswered. The idea to use a PDE-5-inhibitor in portal hypertension has mainly been derived from theoretical consideration based on known or assumed biochemical mechanisms involved in portal hypertension<sup>[16,17]</sup>. Meanwhile, the results of a proof-of-concept study were published showing that the long-acting PDE-5-inhibitor Udenafil lowers portal pressure in liver cirrhosis in a dose dependent-manner in the acute setting<sup>[8]</sup>. The authors tried to explain the conflicting data obtained from other working groups and suggested that the effect of a PDE-5-inhibitor is dose-dependent and that the most pronounced effect could be seen in early to middle stages of liver cirrhosis, when the regulation of the sinusoidal tonus could still be influenced. However, the potential for a beneficial or detrimental effect of PDE-5 inhibitors may depend on the stage of liver disease and the extension of portal collaterals as it has been postulated for nitrates<sup>[18]</sup>.

According to recently published articles, a decrease of HVPG by > 10% after acute administration of propranolol might be sufficient to predict a beneficial clinical effect on rate of rebleeding<sup>[11-13]</sup>. In this case it was shown that using the short acting PDE-5-inhibitor Vardenafil and the long-acting PDE-5-inhibitor Tadalafil the portal pressure decreased by 14% and by 15%, respectively. These data are consistent with other published reports<sup>[4,7]</sup>. It was therefore interesting to monitor the effect of the PDE-5-inhibitor on clinical outcome measures in this patient. It was possible to observe the effect of Tadalafil on HVPG for one year. If a PDE-5-inhibitor lowers portal pressure this may be achieved by dilation of sinusoids which leads to a concomitant increase of portal venous blood flow<sup>[4]</sup>.





**Figure 2** Distal aspect of the esophagus (12/2016). Endoscopy shows scarring in the distal esophagus caused by sclerosing and banding, the old varices remain thrombosed.



**Figure 3** Computer tomographic angiography of the abdomen (09/2017). These sections show the normal sized-cirrhotic liver with the portal vein draining blood in prograde direction (solid arrow). The spleen is considerably enlarged. There is a collateral blood flow originating from the splenic hilum via an enlarged ovarian vein to the pelvis (dashed arrows).

Table 1 shows that the PDE-5-inhibitor induced an increase of portal venous flow. Within a two-year duration this increase was verified by two independent methods (Doppler sonography and MRT).

This first documented case of a long-term application of PDE-5 inhibitors in cirrhotic portal hypertension could initiate the discussion about this group of drugs as novel adjunct therapy in this setting. Further clinical studies must be conducted before PDE-5 inhibitors can be safely recommended for this indication. In this patient the effect of Tadalafil on portal blood flow persisted for eight years. Presently, there is still no generally accepted non-invasive marker for portal pressure<sup>[19]</sup>. The quantification of liver hemodynamics with Doppler sonography has been shown to correlate with the HVPG to some degree<sup>[20,21]</sup>. The effect on portal blood flow may therefore be a further surrogate marker for the influence of a drug on portal pressure in portal hypertension. It can be supposed that an increase of portal flow induced by a PDE-5-inhibitor in liver cirrhosis may indicate a preserved reactivity of the sinusoids and lowering of portal pressure by the drug. However, the reliability of

the sonographic data depends on the expertise of the examiner. In this case, the MRI measurement showed an effect consistent with the Doppler sonographic results. Therefore, it is probable that during the long-term therapy with Tadalafil the positive effect on portal pressure persisted.

Another notable observation was that during the eight years of follow-up no signs of deterioration of liver function were found. AIH, PBC, and AIH/PBC overlap syndrome have unfavourable prognosis once cirrhosis developed and even more after bleeding from esophageal varices<sup>[22-25]</sup>. This raises the question whether PDE-5-inhibitors could improve or stabilize the function in a diseased liver. In thioacetamide-induced liver fibrosis/cirrhosis in animals Sildenafil was shown to induce a reversal of liver damage and fibrosis<sup>[26]</sup>, and Udenafil had a positive effect on degree of fibrosis in rats<sup>[27]</sup>. It may be speculated whether some kind of remodelling in a diseased liver may be induced by long-term administration of a PDE-5-inhibitor by influencing hepatic stellate cells which are suggested as key mediators of fibrosis<sup>[28]</sup>. In the presented case the diagnosis of cirrhosis had been done in 1989. The course of disease was relatively stable under immunosuppressive therapy for 20 years. However, episodes of upper gastrointestinal bleeding occurred. It is unlikely that after such a time period a spontaneous improvement in portal hypertension would occur. Echocardiographic examinations in the year 2009, 2013 and 2017 revealed constant findings. Taken together with stable values of blood pressure and heart rate no significant changes in systemic circulation were detected during the years of follow-up. Therefore, the improvement in portal hemodynamics could be attributed to the application of the PDE-5-inhibitors.

In conclusion, in this case of a female patient with liver cirrhosis due to overlap-syndrome of AIH and primary biliary cholangitis the application of Vardenafil or Tadalafil induced a decrease of portal pressure by 14% and 15%, respectively, and an increase of portal vein blood flow by 28% (Doppler sonography) and 16% (MRT). The use of a long-acting PDE-5-inhibitor



led to a long-lasting improvement of portal blood flow. During the eight years of follow-up no further bleeding from esophageal varices occurred.

## ARTICLE HIGHLIGHTS

### Case characteristics

This is the first report of long-term treatment of portal hypertension with PDE-5 inhibitors.

### Clinical diagnosis

Variceal bleeding in liver cirrhosis due to autoimmune hepatitis.

### Differential diagnosis

Diagnosis of cirrhosis was done 20 years before start of treatment, so the decline in portal hypertension is probably attributable to PDE-5-Inhibitor-treatment rather than spontaneous improvement.

### Laboratory diagnosis

Improvement of portal hypertension was verified by invasive measurement of hepatovenous pressure gradient.

### Imaging diagnosis

MRI and duplex sonographic images verified improvement in portal hemodynamics.

### Pathological diagnosis

Liver cirrhosis was histologically confirmed 20 years prior to PDE-5-Inhibitor treatment.

### Treatment

Oral treatment with Tadalafil 5mg once daily.

### Related reports

Targeting the nitric oxide (NO)-cGMP-pathway in other diseases like pulmonary hypertension or erectile dysfunction.

### Term explanation

Non-selective beta-blockers are the mainstay of medical therapy for portal hypertension in liver cirrhosis.

### Experiences and lessons

Influencing the NO-pathway by the use of PDE-5 inhibitors may have long-term beneficial effects in compensated cirrhosis.

## REFERENCES

- Laleman W, Landeghem L, Wilmer A, Fevery J, Nevens F. Portal hypertension: from pathophysiology to clinical practice. *Liver Int* 2005; **25**: 1079-1090 [PMID: 16343056 DOI: 10.1111/j.1478-3231.2005.01163.x]
- Shah V, Lyford G, Gores G, Farrugia G. Nitric oxide in gastrointestinal health and disease. *Gastroenterology* 2004; **126**: 903-913 [PMID: 14988844]
- Iwakiri Y, Groszmann RJ. Vascular endothelial dysfunction in cirrhosis. *J Hepatol* 2007; **46**: 927-934 [PMID: 17391799 DOI: 10.1016/j.jhep.2007.02.006]
- Deibert P, Schumacher YO, Ruecker G, Opitz OG, Blum HE, Rössle M, Kreisel W. Effect of vardenafil, an inhibitor of phosphodiesterase-5, on portal haemodynamics in normal and cirrhotic liver -- results of a pilot study. *Aliment Pharmacol Ther* 2006; **23**: 121-128 [PMID: 16393289 DOI: 10.1111/j.1365-2036.2006.02735.x]
- Halverscheid L, Deibert P, Schmidt R, Blum HE, Dunkern T, Pannen BH, Kreisel W. Phosphodiesterase-5 inhibitors have distinct effects on the hemodynamics of the liver. *BMC Gastroenterol* 2009; **9**: 69 [PMID: 19765284 DOI: 10.1186/1471-230X-9-69]
- Tandon P, Inayat I, Tal M, Spector M, Shea M, Groszmann RJ, Garcia-Tsao G. Sildenafil has no effect on portal pressure but lowers arterial pressure in patients with compensated cirrhosis. *Clin Gastroenterol Hepatol* 2010; **8**: 546-549 [PMID: 20144739 DOI: 10.1016/j.cgh.2010.01.017]
- Kreisel W, Deibert P, Kupcinskas L, Sumskiene J, Appenrodt B, Roth S, Neagu M, Rössle M, Zipprich A, Caca K, Ferlitsch A, Dilger K, Mohrbacher R, Greinwald R, Sauerbruch T. The phosphodiesterase-5-inhibitor udenafil lowers portal pressure in compensated preascitic liver cirrhosis. A dose-finding phase-II-study. *Dig Liver Dis* 2015; **47**: 144-150 [PMID: 25483910 DOI: 10.1016/j.dld.2014.10.018]
- de Franchis R; Baveno VI Faculty. Expanding consensus in portal hypertension: Report of the Baveno VI Consensus Workshop: Stratifying risk and individualizing care for portal hypertension. *J Hepatol* 2015; **63**: 743-752 [PMID: 26047908 DOI: 10.1016/j.jhep.2015.05.022]
- Bari K, Garcia-Tsao G. Treatment of portal hypertension. *World J Gastroenterol* 2012; **18**: 1166-1175 [PMID: 22468079 DOI: 10.3748/wjg.v18.i11.1166]
- Garcia-Tsao G, Bosch J. Management of varices and variceal hemorrhage in cirrhosis. *N Engl J Med* 2010; **362**: 823-832 [PMID: 20200386 DOI: 10.1056/NEJMra0901512]
- Villanueva C, Aracil C, Colomo A, Hernández-Gea V, López-Balaguer JM, Alvarez-Urturi C, Torras X, Balanzó J, Guarner C. Acute hemodynamic response to beta-blockers and prediction of long-term outcome in primary prophylaxis of variceal bleeding. *Gastroenterology* 2009; **137**: 119-128 [PMID: 19344721 DOI: 10.1053/j.gastro.2009.03.048]
- La Mura V, Abalde JG, Raffia S, Retto O, Berzigotti A, García-Pagán JC, Bosch J. Prognostic value of acute hemodynamic response to i.v. propranolol in patients with cirrhosis and portal hypertension. *J Hepatol* 2009; **51**: 279-287 [PMID: 19501930 DOI: 10.1016/j.jhep.2009.04.015]
- de-Madaria E, Palazón JM, Hernández FT, Sánchez-Paya J, Zapater P, Irurzun J, de España F, Pascual S, Such J, Sempere L, Carnicer F, García-Herola A, Valverde J, Pérez-Mateo M. Acute and chronic hemodynamic changes after propranolol in patients with cirrhosis under primary and secondary prophylaxis of variceal bleeding: a pilot study. *Eur J Gastroenterol Hepatol* 2010; **22**: 507-512 [PMID: 20150817 DOI: 10.1097/MEG.0b013e32832ca06b]
- Stankovic Z, Csatori Z, Deibert P, Euringer W, Blanke P, Kreisel W, Abdullah Zadeh Z, Kallfass F, Langer M, Markl M. Normal and altered three-dimensional portal venous hemodynamics in patients with liver cirrhosis. *Radiology* 2012; **262**: 862-873 [PMID: 22357888 DOI: 10.1148/radiol.11110127]
- Stankovic Z. Four-dimensional flow magnetic resonance imaging in cirrhosis. *World J Gastroenterol* 2016; **22**: 89-102 [PMID: 26755862 DOI: 10.3748/wjg.v22.i1.89]
- Davies NA, Hodges SJ, Pitsillides AA, Mookerjee RP, Jalan R, Mehdizadeh S. Hepatic guanylate cyclase activity is decreased in a model of cirrhosis: a quantitative cytochemistry study. *FEBS Lett* 2006; **580**: 2123-2128 [PMID: 16563392 DOI: 10.1016/j.febslet.2006.02.080]
- Loureiro-Silva MR, Iwakiri Y, Abalde JG, Haq O, Groszmann RJ. Increased phosphodiesterase-5 expression is involved in the decreased vasodilator response to nitric oxide in cirrhotic rat livers. *J Hepatol* 2006; **44**: 886-893 [PMID: 16545481 DOI: 10.1016/j.jhep.2006.01.032]
- Angelico M, Lionetti R. Long-acting nitrates in portal hypertension: to be or not to be? *Dig Liver Dis* 2001; **33**: 205-211 [PMID: 11407662]
- Bolognesi M, Di Pascoli M, Sacerdoti D. Clinical role of non-invasive assessment of portal hypertension. *World J Gastroenterol* 2017; **23**: 1-10 [PMID: 28104976 DOI: 10.3748/wjg.v23.i1.1]
- Iranpour P, Lall C, Houshyar R, Helmy M, Yang A, Choi JI, Ward G, Goodwin SC. Altered Doppler flow patterns in cirrhosis

- patients: an overview. *Ultrasonography* 2016; **35**: 3-12 [PMID: 26169079 DOI: 10.14366/usg.15020]
- 21 **Tasu JP**, Rocher L, PEletier G, Kuoch V, Kulh E, Miquel A, Buffet C, BIEry M. Hepatic venous pressure gradients measured by duplex ultrasound. *Clin Radiol* 2002; **57**: 746-752 [PMID: 12169287]
  - 22 **Imam MH**, Lindor KD. The natural history of primary biliary cirrhosis. *Semin Liver Dis* 2014; **34**: 329-333 [PMID: 25057955 DOI: 10.1055/s-0034-1383731]
  - 23 **Floreani A**, Franceschet I, Cazzagon N. Primary biliary cirrhosis: overlaps with other autoimmune disorders. *Semin Liver Dis* 2014; **34**: 352-360 [PMID: 25057958 DOI: 10.1055/s-0034-1383734]
  - 24 **European Association for the Study of the Liver**. EASL Clinical Practice Guidelines: Autoimmune hepatitis. *J Hepatol* 2015; **63**: 971-1004 [PMID: 26341719 DOI: 10.1016/j.jhep.2015.06.030]
  - 25 **Kirstein MM**, Metzler F, Geiger E, Heinrich E, Hallensleben M, Manns MP, Vogel A. Prediction of short- and long-term outcome in patients with autoimmune hepatitis. *Hepatology* 2015; **62**: 1524-1535 [PMID: 26178791 DOI: 10.1002/hep.27983]
  - 26 **Said E**, Said SA, Gameil NM, Ammar EM. Modulation of thioacetamide-induced liver fibrosis/cirrhosis by sildenafil treatment. *Can J Physiol Pharmacol* 2013; **91**: 1055-1063 [PMID: 24289076 DOI: 10.1139/cjpp-2013-0181]
  - 27 **Choi SM**, Shin JH, Kim JM, Lee CH, Kang KK, Ahn BO, Yoo M. Effect of udenafil on portal venous pressure and hepatic fibrosis in rats. A novel therapeutic option for portal hypertension. *Arzneimittelforschung* 2009; **59**: 641-646 [PMID: 20108650 DOI: 10.1055/s-0031-1296453]
  - 28 **Cohen-Naftaly M**, Friedman SL. Current status of novel antifibrotic therapies in patients with chronic liver disease. *Therap Adv Gastroenterol* 2011; **4**: 391-417 [PMID: 22043231 DOI: 10.1177/1756283X11413002]

**P- Reviewer:** Gallo P, Karagiannakis DS, Konishi H, Risso A

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ISSN 1007-9327

